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METABOLISM OF DIMETHYLSULFIDE IN THE *Bacteria*

by

Rich Boden B.Sc (*Lond.*)

THIS THESIS IS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF BIOLOGICAL SCIENCES

UNIVERSITY OF WARWICK, COVENTRY, UK.

TO MY FRIENDS & FAMILY

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DECLARATION

THE WORK CONTAINED IN THIS THESIS IS THE RESULT OF ORIGINAL RESEARCH BY MYSELF UNDER THE SUPERVISION OF DR HENDRIK SCHÄFER AND PROF J C MURRELL EXCEPTING THE FOLLOWING:

(A) STABLE-ISOTOPE PROBING EXPERIMENTS (CHAPTER 6) WERE PERFORMED IN CONJUCTION WITH DR J D NEUFELD, UNIVERSITY OF WARWICK, UK.

(B) LCI-ESI-MS/MS ANALYSES OF PROTEINS WERE PERFORMED BY MISS N PATEL, MISS E Q BLATHERWICK AND MS S E SLADE, PROTEOMICS FACILITY, UNIVERSITY OF WARWICK.

ALL SOURCES OF INFORMATION HAVE BEEN ACKNOWLEDGED BY MEANS OF REFERENCES.

NONE OF THE WORK CONTAINED IN THIS THESIS HAS BEEN SUBMITTED FOR ANY PREVIOUS DEGREE.

RICH BODEN
MAY 2009

ACKNOWLEDGEMENTS

Firstly, I would like to thank my Supervisors, Dr Hendrik Schäfer and Prof J Colin Murrell for their unending support, encouragement and patience during this study. I also owe a debt of thanks to Prof Donovan P Kelly for support, advice and encouragement.

I would like to thank Dr Ann P Wood (King's College London, UK) and Dr Elena Borodina (University of Bristol, UK) for allowing access to unpublished data on *Hyphomicrobium sulfonivorans, Methylobacterium podarium* and *Xanthobacter tagetidis*; Dr Paul R Norris (University of Warwick, UK) for the loan of the Warburg apparatus, use of the atomic absorption spectrometer and for many stimulating discussions on sulfur metabolism; Dr Nataliya Myronova and Ms Susan E Slade (University of Warwick, UK) for assistance with respect to protein purification; Mr Gez Chapman, Mrs Jane Green, Mrs Jenny Flint and Mrs Annora Johnson (University of Warwick, UK) for helping to track down various pieces of obscure apparatus and for technical support; Dr Lesley Robertson (Technische Universiteit Delft, Netherlands) for the provision of copies of papers and monographs from the Delft archives; Dr Larry Kupchella (Indiana University of Pennsylvania, USA) for stimulating discussions with respect to polythionate analysis; Prof Yuri Trotsenko (Institute of Biochemistry and Physiology of Microorganisms, Russia) for the provision of *Methylophaga* spp.

Members of the Schäfer, Murrell and Norris groups (past and present) in particular Marc, Jo, Elena, Michael, Sanjeev, Jimmy, Josh, Carly, Chen, Deepak, Julie, Hélène, Aurelie, Marina *et al.*!

Away from the lab, I'd like to thank a number of people for support and encouragement over the past few years but, since they should know who they are, they shall retain their anonymity here.

ABBREVIATIONS & ACRONYMS

α – Bunsen coefficient
ε – Molar extinction coefficient
λ - Wavelength
μ - Specific growth rate
μ_{MAX} – Maximum specific growth rate
ρ – Density
χ - Volume of gas evolved/absorbed
a – Specific maintenance rate
A_x – Absorbance at x nm
AAS – Atomic absorption spectroscopy
ACN - Acetonitrile
ADP – Adenosine 5'-diphosphate
AMP – Adenosine 5'-monophosphate
Amitrole – 3-amino-1,2,4-triazole
AOA – Antioxidative activity
APS – Adenylyl sulfate
ATP – Adenosine 5'-triphosphate
BIS - 2,2-Bis(hydroxymethyl)-2,2',2''-nitrotriethanol
BLAST – Basic local alignment search tool
BSA – Bovine serum albumin
BV – Benzyl viologen
CBS – Complete basal salts
CBB cycle – Calvin-Benson-Bassham cycle
CCCP – Carbonylcyanide-*p*-chlorophenylhydrazone
CFE – Cell-free extract
CLAW hypothesis – Charlson-Lovelock-Andreae-Warren hypothesis
CMB – *p*-hydroxymercuribenzoate
CTAB – Cetyl trimethylammonium bromide
Cyt – Cytochrome
D – Dilution rate
Da – Dalton
DBT - Dibenzothiophene
DCCD – *N*,*N*'-dicyclohexylcarbodiimide
DCPIP – 2,6-Dichlorophenolindophenol
DGGE – Denaturing-gradient gel electrophoresis
DMA – Dimethylamine
DMDS - Dimethyldisulfide
DMS – Dimethylsulfide
DMSO – Dimethylsulfoxide
DMSO$_2$ – Dimethylsulfone
DMSP – β-Dimethylsulfoniopropionate
DMTS – Dimethyltrisulfide
DMTTS - Dimethyltetrasulfide

DNA – Deoxyribonucleic acid
DNAse - Deoxyribonuclease
DNP – 2,4-dinitrophenol
DPI – Diphenyleneiodonium chloride
DTT – Dithiothreitol
e – Electron
E_G – Growth efficiency
EC number – Enzyme Commission number
EDTA – Ethylenediamine tetraacetic acid
EVS – Ethyl vinyl sulfide
FBP – Fructose-1,6-bisphosphate
FCCP – Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
FMN – Flavin mononucleotide (oxidised form)
$FMNH_2$ – Flavin mononucleotide (reduced form)
g – Nominal acceleration due to gravity at sea-level
GC – Gas chromatography
GOS project – Global ocean sequencing project
GYB – Glucose-yeast-beef medium
h - hours
h – Change in height of manometer fluid
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC – High-performance liquid chromatography
HQNO – 8-hydroxy-quinoline-*N*-oxide
IA - Iodoacetamide
$\mathcal{K}$ – Flask constant
k_C – Coupling constant
k_M – Michaelis constant
k_S – Saturation constant
KDPG – 2-keto-3-deoxy-6-phosphogluconate
m – Maintenance energy coefficient
m_S – Maintenance energy coefficient with respect to substrate
m_{ATP} – Maintenance energy coefficient with respect to ATP
M - Molar
MAMS – Marine ammonium mineral salts
MDR – Mixed diamine reagent
Mercurochrome™ - Dibromohydroxymercurifluorescein
Mersal - 2-(3-hydroxymercurio-2-methoxypropylcarbamoyl)-phenoxyacetate
MF – Manometer fluid
MMA – Monomethylamine
Mol – Mole
Mol% - Molar percentage
MP – Melting point
MSA – Methanesulfonate
MT – Methanethiol
MTBE – Methyl *tert*-butyl ether
MV – Methyl viologen

M_W – Molecular weight
MWCO – Molecular weight cut-off
n – Number of moles
NAD$^+$ - Nicotinamide adenine dinucleotide (oxidised form)
NADH – Nicotinamide adenine dinucleotide (reduced form)
NADP$^+$ - Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH – Nicotinamide adenine dinucleotide phosphate (reduced form)
N.D. – Not determined
NEM – *N*-ethylmaleimide
NTA - Nitrilotriacetate
OD_x – Optical density at x nm
P/2*e* ratio – Molar ratio of phosphorylation per electron pair
P/O ratio – Molar oxidative phosphorylation ratio
P_0 – Standard pressure
PCA – Perchloric acid
PCR – Polymerase chain reaction
PGA – 3-phosphoglycerate
PIPES – 1,4-Piperazinediethanesulfonic acid
PMS – Phenazine methosulfate
PSO – *Paracoccus* sulfur oxidation
PTFE – Polytetrafluoroethylene
Purpald® - 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole
PVDF - Polyvindylidene difluoride
q – Specific rate of substrate uptake
RNA – Ribonucleic acid
RNAse - Ribonuclease
RPM – Revolutions per minute
RuBisCO – Ribulose bisphosphate carboxylase:oxygenase
RuMP – Ribulose monophosphate
S_4I – Tetrasulfur intermediate
SA – Specific activity
SDS – Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIP – Stable-isotope probing
SMM – *S*-methylmethionine
^{T} – Type strain
T2A – Thiophene-2-acetate
T2MA – Thiophene-2-methylamine
T3A – Thiophene-3-acetate
T2C – Thiophene-2-carboxylate
T3C – Thiophene-3-carboxylate
TE buffer – 100mM Tris-HCl pH 8.0 + 1mM EDTA
Thiometon – *O,O*-dimethyl phosphorodithioate
Thorin - 3-hydroxy-4-[(E)-(2-arsonophenyl)diazenyl]naphthalene-2,7-disulfonate
TLC – Thin-later chromatography
TMA – Trimethylamine

TOMES – Thiosulfate-oxidising multi-enzyme system
Tricine – *N*-[Tris(hydroxylmethyl)methyl]glycine
Tris – 2-amino-2-hydroxymethyl-1,3-propanediol
Triton® X-100 - Polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether
Tween® 80 – (*x*)-sorbitan mono-9-octadecanoate poly(oxy-1,2-ethanediyl))
UQ – Ubiquinone
V – Rate of reaction
V_{MAX} – Maximum rate of reaction
V_{f} – Volume of liquid in manometer flask
V_{g} – Volume of gas in manometer system
VOSC – Volatile organosulfur compound
v/v – Volume to volume
w/v – Weight to volume
x – Dry weight of biomass
Y – Yield
Y_{ATP} – Yield of ATP
Y_{E} – Yield with respect to energy source
Y_{G} – Theoretical molar growth yield
Y_{MAX} – Maximum yield
$Y_{\mathrm{X/S}}$ – Yield with respect to grams of dry biomass formed per gram of substrate
ZAV – Zinc amalgam-vanadium reducing reagent

SUMMARY

Dimethylsulfide (DMS) is a volatile organosulfur compound which has been implicated as playing key roles in climate control and in the biogeochemical cycling of sulfur. Metabolism of DMS by *Bacteria* has been previously identified as an important sink of DMS in soils and in the marine environment; however, relatively little is known about the physiology or biochemistry of *Bacteria* that metabolism DMS.

The key enzyme of DMS oxidation in *Hyphomicrobium* spp. – DMS monooxygenase - has been purified and characterised from *H. sulfonivorans*. It has been shown to be a two-component monooxygenase, related to bacterial luciferase, comprising two subunits – an $FMNH_2$-dependent DMS monooxygenase (DmoA) and an NADH-dependent FMN oxidoreductase (DmoB). For DMS, DMS monooxygenase from *H. sulfonivorans* has a V_{max} of 1250 nmol DMS oxidised min^{-1} (mg protein)$^{-1}$ and a k_M of 16.5μM, corresponding to a k_{CAT} of 5.2s^{-1}.

DMS oxidation in terms of acting as a sole-carbon source and as a supplementary energy source has been demonstrated in methylotrophic and heterotrophic bacteria. Chemolithoheterotrophic growth in which DMS carbon is assimilated to biomass whilst DMS sulfur is oxidised to tetrathionate with a net energy gain has been demonstrated in "*M. thiooxidans*". Both "internal" and "external" chemolithoheterotrophy has been observed in "*M. thiooxidans*", with endogenous and exogenous thiosulfate being oxidised to tetrathionate with a net energy gain. As far as can be found from the literature, this is the first recorded production of a

polythionate from an organosulfur compound, as such, representing a potential new step in the biogeochemical sulfur cycle.

Stable-isotope probing with [$^{13}C_2$]-DMS has been performed for the first time and has confirmed *Methylophaga* spp. as dominant DMS-oxidising *Bacteria* in the marine environment. The oxidation of marine thiosulfate to tetrathionate has been demonstrating during a phytoplankton bloom, indicating that chemolithoheterotrophic *Bacteria* are active during the bloom.

Preliminary analyses have been carried out on the genome sequence of "*Methylophaga thiooxidans*" and the genes encoding the major enzymes of formaldehyde assimilation *via* the KDPG aldolase variant RuMP pathway have been identified. Genes encoding key enzymes involved in the dissimilation of methanol and methylated amines have been indentified, in addition to those involved in nitrogen uptake from ammonia, nitrate, nitrite and urea.

Chemoorganoheterotrophic growth, coupling the oxidation of DMS to DMSO with ATP formation, has been demonstrated in *Sagittula stellata* E-37^{T}, though the enzyme(s) responsible for this oxidation remain unclear.

"*Es erhebt sich nun die Frage, was unter den natürlichen Lebensbedingungen dieser Organismen die Ausgangs- und Endproduckte ihres Stoffwechsel sind. Leider lässt sie sich mit voller Bestimmtheit nicht beantworten*"

Dr Alexander Nathansohn (*fl.* 1900)

Mitt. zool. Stn. Neapel. **15**: 655-680 (1902).

CHAPTER 1
INTRODUCTION

1.1 The biogeochemical sulfur cycle

Sulfur occurs in nature in a diverse range of organic and inorganic compounds which can be transformed by a variety of chemical and biochemical reactions providing a constant flux of sulfur compounds between different environments – the sulfur cycle (reviewed by Loka Bharathi 2008).

The sulfur cycle is perhaps the least understood of the major biogeochemical cycles as it has a greater inherent complexity than those of carbon or nitrogen due to the wide range of oxidation states in which sulfur can exist: -2 to +6 (Kelly 1988). The general process of the sulfur cycle can be regarded at a basic level as the oxidation of sulfide to sulfate in oxic environments and the reduction of sulfate to sulfide in anoxic environments (Eriksson 1963). It should be noted that there is a vast array of sub-cycles within the biogeochemical sulfur cycle – many of which are microbially driven – which are poorly understood.

Volatile organosulfur compounds (VOSCs) are recognised as playing important roles in the sulfur cycle, owing to the facility of their transfer from solution to the gas phase and *vice versa*. The low molecular-weight VOSCs, such as dimethylsulfide (DMS), dimethyldisulfide (DMDS), methanethiol (MT), carbon disulfide (CS_2) and carbonyl sulfide (COS) are recognised as being of particular importance with regard to microbially driven sulfur cycling (Smith 1988). It is worth noting that CS_2 and COS are usually classed as inorganic sulfur compounds but they are often grouped with the VOSCs since they have similar biogeochemical roles.

1.2 Dimethylsulfide

Dimethylsulfide (DMS) is a colourless, volatile, flammable liquid (MP = -98°C, BP = 37°C) with a pungent and penetrating odour (Ljunggren & Norberg 1948; Leach & Chung 1982) with the formula $(CH_3)_2S$. DMS is considered to be the most prevalent VOSC in the atmosphere in terms of throughput (COS is more prevalent in terms of steady-state concentrations; Simpson *et al.* 1999); however, prior to 1978, hydrogen sulfide (H_2S) was considered to be more prevalent (Bremner & Steele 1978). DMS as a pure compound has a disagreeable odour akin to that of rotting cabbage; however, it takes on a more pleasant smell in low concentrations and is often described as having "the smell of the sea" (Malin *et al.* 1992). It should be noted that DMS makes up only a minor part of the odour-profile of the oceans – the major components being dictyopterenes produced by phytoplankton (Kajiwara 1983).

1.2.1 Effects of DMS on the environment

DMS has been implicated as a climate-controlling gas in the so-called "CLAW" hypothesis, named for the initials of the authors of the original paper (Charlson *et al.* 1987). The hypothesis falls within the Gaia hypothesis of global feedback (Baas Becking 1931, Lovelock & Margulis 1974) and states that DMS produced in the oceans (following the death of phytoplankton) is volatilised into the lower atmosphere where it is photochemically oxidised to various species including methanesulfonate (MSA), sulfur dioxide (SO_2), sulfate (SO_4^{2-}), which can assist in the formation of high-albedo clouds, which reflect sunlight. According to the hypothesis, increased reflection of sunlight results in – ignoring all other factors - decreased growth of phototrophic phytoplankton, therefore, decreased DMS formation, leading to a negative feedback loop.

The so-called anti-CLAW hypothesis (Lovelock 2007) has also been proposed, in which global warming results in higher ocean temperatures, which decreases phytoplankton growth, thus reducing DMS, MSA, SO_2 and SO_4^{2-} concentrations in the lower atmosphere, resulting in a decrease in the formation of high-albedo clouds and therefore an increase in global warming. Since DMS is readily volatilised from solution in seawater and gaseous DMS is soluble in rain water, it has been postulated that DMS provides a shunt within the sulfur cycle, transporting sulfur from aquatic to terrestrial environments (Nguyen *et al.* 1978).

1.2.2 Transformations in the biogeochemical cycling of DMS

The main chemical transformations of DMS in the environment are photooxidation reactions, resulting in the formation of MSA, SO_4^{2-}, SO_2, sulfur trioxide (SO_3), dimethylsulfoxide (DMSO) and dimethylsulfone ($DMSO_2$) in addition to formaldehyde (HCHO).

1.2.3 Sources of DMS

DMS is produced by a variety of chemical and biological processes, both naturally and anthropogenically.

1.2.3.1 Anthropogenic sources of DMS

The major anthropogenic sources of DMS are the paper, brewing and agricultural industries (Leach & Chung 1982; Hansen 1999, Vitenberg *et al.* 2007). The major source of DMS in the Kraft paper process and petrochemical industries is as a biproduct of the Swern oxidation of alcohols to aldehydes (Omura & Swern 1978). DMS is also produced, along with other VOSCs in the farming industry, particularly in fowl hatchery halls (Tymczyna *et al.* 2007) and in swine farms (Kim KY *et al.* 2007). Emissions from the farming industry are thought to be the result of the metabolism of facultative anaerobic *Bacteria* and *Archaea* in anoxic zones within blackwater and excrement heaps (Lens *et al.* 1995). Associated with the farming industry are animal rendering plants, in which slaughter-house waste is converted into products such as bonemeal and tallow. Such plants heat slaughter-house waste to 105°C, in which Maillard reactions result in the production of DMS, along with significant amounts of other VOSCs, amines and aldehydes (van Langenhove *et al.* 1982). High-temperature processes such as the heat-treatment of sewage and in the food industry also result in the release of DMS, VOSCs and thiophenes (Schamp & van Langenhove 1986).

1.2.3.2 Terrestrial biogenic sources

DMS is produced by various *Eukarya* including fungi, plants and animals (Riemenschneider 2006; Schroeder 1993). A list of DMS-producing species is given in Table 1.1. Due to its prevalence in a wide range of plants which form part of the human diet, extensive studies into DMS as a flavour-component in foods have been undertaken (Bentley & Chasteen 2004). DMS has been found, along with thiols and substituted thiophenes in roast meats (Rochat *et al.* 2007), the formation of which has been demonstrated from Maillard reactions of sulfur-containing proteins with sugars and thiamine (Cerny 2007). This would appear to indicate that burning animal and plant matter, such as during deforestation, could contribute DMS (along with other VOSCs) to the atmosphere

Latin name	Common name	Reference
Allium jesdianum Boiss. & Buhse	Persian garlic	Amiri 2007
Allium sativum L.	Garlic	Lawson *et al.* 1991
Amorphophallus albispathus Hett.	-	Kite & Hetterschieid 1997
Brassica oleraceae var. *botrytis* L.	Cauliflower	Engel *et al.* 2002
Camelia sinensis L.	Tea	Smet *et al.* 1998
Capsicum annuum L.	Bell pepper	Cremer & Eichner 2000
Coffea arabica L.	Arabica coffee	Schamp & van Langenhove 1986
Diospyros kaki L.	Persimmon	Geng & Mu 2006
Fragaria vesca Cov.	Woodland strawberry	Jetti *et al.* 2007
Helicodiceros muscivorus Engl.	Dead-horse arum lily	Stensmyr *et al.* 2002
Homo sapiens L.	Human	Suarez *et al.* 1998
Hydnora africana Thunb.	Jackal food	Burger *et al.* 1988
Platanus occidentalis L.	Buttonwood	Geng & Mu 2006
Platanus orientalis L.	Oriental plane	Geng & Mu 2006
Populus canadensis Moench	Canadian poplar	Geng & Mu 2006
Salix babylonica L.	Peking willow	Geng & Mu 2006
Solanum lycopersicum L.	Tomato	Williams 1973
Styphnolobium japonicum Schott	Pagoda tree	Geng & Mu 2006
Theobroma cacao L.	Cacao	Smet *et al.* 1998
Trigonella foenum-graecum L.	Fenugreek	Mebazaa *et al.* 2009
Tuber magnatum	White truffle	Bellesia *et al.* 1996
Tuber megasporum	Black truffle	Talou *et al.* 1990
Zea mays var. *rugosa* L.	Sweetcorn	Bills & Keenan 1968

Table 1.1 Terrestrial *Eukarya* known to produce DMS.

1.2.3.3 Marine biogenic sources: β-dimethylsulfoniopropionate

DMS is produced in the marine environment by the metabolism of the algal osmolyte β-dimethylsulfoniopropionate (DMSP) by *Bacteria* (Bremner & Steele 1978). DMSP was first isolated in 1967 from *Crypthecodinium cohnii* (previously *Gyrodinium cohnii*) as "dimethyl-β-propiothetin" (Ishida & Kadota 1967); however, relatively little of the biochemistry and physiology of DMSP anabolism and catabolism has been understood until the last ten years.

DMSP is a sulfur-analogue of betaine and is, like betaine, thought to provide a stabilising effect on proteins during osmotic extremes. DMSP has been shown to stabilise key metabolic enzymes such as glucose-6-phosphate dehydrogenase and malate dehydrogenase in *Tetraselmis subcordiformis* Stein, a member of the *Chlorophyceae* (Gröne & Kirst 1991). DMSP concentrations in salt-shocked algal cells can reach as high as 0.1M (Mason & Blunden 1989; Dickson *et al.* 1980).

DMSP is produced in salt-tolerant plants such as *Wollastonia biflora* DC., *Spartina anglica* Hubb. (Common cordgrass) and *Saccharum* spp. L. (Sugar cane) as a shock-response to increases in salinity (Trossat *et al.* 1998; Paquet *et al.* 1994; Larher *et al.* 1977). DMSP is synthesised in the chloroplasts from *S*-methylmethionine (SMM) which is synthesised in the cytosol from methionine (Trossat *et al.* 1996). Synthesis of DMSP from SMM is *via* the intermediate dimethylsulfoniopropionaldehyde, which has been shown to spontaneously decompose to yield DMS and acrylaldehyde – allowing DMS to be produced directly from salt-tolerant plants independently of any bacterial lysis of DMSP (James *et al.* 1995).

The pathways of DMS production from DMSP were, until recently, poorly understood; however, it is now clear that a range of bacterial enzymes facilitate the production of DMS from DMSP. The dominant pathway of bacterial DMSP degradation does not, in fact, result in the formation of DMS but in that of MT. DMSP demethylase (DmdA), found in members of the *Alphaproteobacteria* such as *Ruegeria pomeroyi* (previously *Silicibacter pomeroyi*) and members of the SAR11 clade (Howard *et al.* 2006). A second pathway of DMSP degradation by *Bacteria* results in the liberation of DMS – the DMSP lyase pathway – encoded by the *ddd* gene cluster. DMSP lyase is found in members of the *Gammaproteobacteria* such as *Oceanimonas doudoroffii* (previously *Pseudomonas doudoroffii*) and in members of the *Betaproteobacteria* such as *Alcaligenes* spp. (de Souza & Yoch 1996) and algae such as *Ulva curvata* (de Souza *et al.*1996). Homologs of the *ddd* genes have been found in members of *Alphaproteobacteria* such as *Fulvimarina* spp., *Loktanella* spp., *Oceanicola* spp., *Stappia* spp., *Sulfitobacter* spp., *Sagittula* spp. and *Rhizobium* spp., along with members of the *Betaproteobacteria* such as *Burkholderia capacia* (Curson *et al.* 2008). A third pathway, involving an acyltransferase has been described in *Marinomonas* spp. (Todd *et al.* 2007).

DMS is produced from DMSP in marine sponges (*Ircinia* spp. Duch. & Mich.; Pawlik *et al.* 2002). Coral reefs produce large amounts of DMS, and the concentrations in their locale are amongst the highest recorded (Broadbent &

Jones 2004). Shellfish such as *Mytilus edulis* L. (Blue mussel), *Argopecten irradians* Mont. (Atlantic bay scallop) and *Crassostrea gigas* Thunberg (Pacific oyster) are also major producers of DMS, caused by the enzymatic lysis of DMSP in ingested phytoplankton (Hill & Dacey 2007; Piveteau *et al.* 2000).

1.2.4 Metabolism of DMS

The first organisms identified to oxidise DMS were *Thiobacillus* spp. isolated from a *Pinus* sp. L. (Pine) bark biofilter at a paper mill (Sivelä & Sundman 1975). Isolates obtained in this study were capable of using DMS at concentrations of up to 2.4mM as an energy (and possibly carbon) source during mixotrophic growth. It is worth noting that DMS-oxidation and assimilation of DMS-carbon to biomass was found in *Klebsiella pneumoniae* (previously *Aerobacter aerogenes*; Rammler & Zaffaroni 1967) during studies into the effects of DMSO on bacterial growth, but this was not identified as such at the time. Isolation of DMS-oxidising organisms has been identified as problematic, due to the toxicity of DMS (Scheulderman-Suylen *et al.* 1985), and, as such, many DMS-oxidising *Bacteria* were isolated from enrichment cultures grown on less toxic substrates such as DMSO (de Bont *et al.* 1981) or $DMSO_2$ (Borodina *et al.* 2000) with comparatively few validated strains having been isolated on DMS itself (Smith 1987). A comprehensive list of *Bacteria* capable of growth on DMS as a sole carbon source is given in Table 1.2. The majority of isolates capable of using DMS as a carbon and energy source are members of the *Alphaproteobacteria* and *Betaproteobacteria*, specifically *Hyphomicrobium* spp. and *Thiobacillus* spp., with a few members of the *Gammaproteobacteria*, namely *Methylophaga* spp., representing the marine isolates. A small number of Gram positive organisms, including *Arthrobacter* spp., *Micrococcus luteus* and *Bacillus licheniformis* have been isolated which can use DMS as a carbon and energy source and have been demonstrated to use standard enzymes of methylotrophic carbon assimilation when growing on DMS, thus indicating that methylotrophy – and oxidation of C_1-sulfur compounds in particular – is perhaps more widespread than previously expected (Anesti *et al.* 2005, Boden *et al.* 2008).

The bacterial metabolism of DMS can be divided into organisms which use DMS as a carbon and energy source, assimilating the carbon to biomass (either *via* the ribulose monophosphare (RuMP) pathway or serine cycle in the case of methylotrophs or *via* the Calvin-Benson-Bassham (CBB) cycle in the case of autotrophs); organisms which oxidise DMS to DMSO as an energy source, without assimilation of carbon into biomass and organisms which use DMS as a sulfur source. With respect to use of DMS as an energy source, the key enzyme is DMS dehydrogenase, which is a three-subunit, molybdenum-containing protein (McDevitt *et al.* 2002), characterised in the purple non-sulfur bacterium *Rhodovulum sulfidophilum* (previously *Rhodopseudomonas sulfidophila* and *Rhodobacter sulfidophilus*).

In terms of DMS as a carbon and energy source, two pathways have been suggested – one containing DMS monooxygenase and one containing DMS methyltransferase (de Bont *et al.* 1981; Visscher & Taylor 1993a).

DMS oxidation *via* the monooxygenase pathway consists of an initial NAD(P)H-dependent oxidation by DMS monooxygenase to MT and formaldehyde (de Bont *et al.* 1981):

$$(CH_3)_2S + NAD(P)H + O_2 \rightarrow CH_3SH + HCHO + NAD(P)^+ + H_2O$$

DMS monooxygenase is thought to be inhibited by methyl *tert*-butyl ether (MTBE) and enzyme activities in DMS-grown cells are generally low – apparently due to the low stability of the enzyme (Visscher & Taylor 1993a).

MT is then oxidised by methanethiol oxidase to yield sulfide and formaldehyde:

$$CH_3SH + H_2O + O_2 \rightarrow HCHO + S^{2-} + H_2O_2$$

It should be noted that certain forms of MT oxidase (namely those from *Hyphomicrobium* spp.) can oxidise sulfide to sulfite (Suylen *et al.* 1987). Toxic hydrogen peroxide is reduced to water and oxygen by catalase and formaldehyde molecules are either assimilated to biomass (*via* the serine or RuMP pathways) or converted to carbon dioxide (*via* formate). In the case of DMS-utilising *Thiobacillus* spp., carbon is assimilated from carbon dioxide *via* the CBB cycle. Sulfide is oxidised to sulfite (either by methanethiol oxidase, as is probably the in the case in *Hyphomicrobium* spp. or by a sulfide oxygenase, in the case of *Thiobacillus* spp.), which is then oxidised to sulfate (*via* sulfite oxidase). Methanethiol oxidase can be inhibited by cyanide, *N*-ethylmaleimide (NEM) and sulfide (Gould & Kanagawa 1992).

The putative DMS methyltransferase pathway differs from the DMS monooxygenase pathway only in the first step. DMS is demethylated to MT *via* the oxygen-independent DMS methyltransferase (Visscher & Taylor 1993a, b):

$$(CH_3)_2S + X\text{-}H \rightarrow CH_3SH + X\text{-}CH_3$$

The methyl- group from DMS is transferred onto a carrier ("X") and then assimilated to biomass after transfer into the serine or RuMP pathways. Though the nature of the carrier co-factor is unknown, it has been suggested that it is cobalamin-related, as is the case in the methyltransferases found in methanogenic *Archaea* and methyl halide-utilising *Bacteria* (Visscher & Taylor 1993a, b).

"Gratuitous" oxidation of DMS to DMSO in heterotrophic *Bacteria* with no noted energy gain has been described in *Delftia acidovorans* (previously *Pseudomonas acidovorans*; Zhang L *et al.* 1991) and in *Sagittula stellata* (Gonzalez *et al.* 1997). It is worth noting that the seemingly "gratuitous" oxidation of DMS was observed in batch cultures, in which, of course, the organism is not limited by carbon source and would not increase in yield in the presence of an auxiliary energy source. To confirm whether or not the oxidation is truly gratuitous, experiments would need to be conducted using susbtrate-limited chemostat culture. The DMS oxidation in *D. acidovorans* has been shown to be NADPH-dependent and particularly rapid in cells grown on 23mM gluconate as sole carbon and energy source (Zhang L *et al.* 1991). A comprehensive list of *Bacteria* able to oxidise DMS without assimilation of the carbon or sulfur into biomass is given in Table 1.3. Oxidation of DMS to DMSO has been demonstrated in various *Eukarya* including *Dunaliella* spp., *Spinacia oleracea* L. (Spinach), *Allium fistulosum* L. (Welsh onion), *Ulva pertusa* L. (Sea lettuce) and *Sargassum* spp. Yendo (Fuse *et al.* 1997). Interestingly, DMS oxidation in such organisms would appear to be pigment-dependent and linked to the presence of pheophytin *a* in DMS-oxidising plants and algae. The same electron carrier is found in some *Bacteria* capable of oxidising DMS to DMSO, including *Thiocapsa roseopersicina* (Jonkers 1999).

DMS can act as an energy source for methanogenic archaea, during which DMS is oxidised to methane and carbon dioxide – this carbon dioxide can then be assimilated to biomass by the organism (Zinder & Brock 1978a).

DMS can act as a sole sulfur source for a wide variety of organisms, ranging from *Bacteria* such as *Pseudomonas putida* (Endoh *et al.* 2005), *Archaea* such as *Methanococcus* spp. (Rajagopal & Daniels 1986) and *Eukarya* such as *Chlorella fusca* (Kraus & Schmidt 1987).

Species	Strain	$[DMS]_{MAX}$	Isolated from	Isolation substrate (concentration)	Reference
Klebsiella pneumoniae[1]	ATCC 9621	*N.D.*	Unknown[2]	Unknown	Rammler & Zaffaroni 1967
Thiobacillus sp.	MS1	2.4mM	*Pinus* sp. bark biofilter from a cellulose mill.	DMS (1.6mM)	Sivelä & Sundman 1975
Hyphomicrobium sp.	S	*N.D.*	Soil (Wageningen, Netherlands)	DMSO (12.8mM)	De Bont *et al*. 1981
Thiobacillus thioparus	Tk-m	2mM	Activated sludge	Thiometon (6mM)	Kanagawa *et al*. 1982; Kanagawa & Kelly 1986
Hyphomicrobium sp.	EG	0.1mM	Papermill biofilter	DMSO (10mM)	Suylen & Kuenen 1986
Thiobacillus sp.	E1	2mM	Commercial peat	DMS (2mM)	Smith 1987
Thiobacillus sp.	E3	2mM	Garden compost	DMS (2mM)	Smith 1987
Thiobacillus sp.	E4	2mM	Cattle manure	DMS (2mM)	Smith 1987
Thiobacillus sp.	E5	2mM	Marine mud (Plymouth, UK)	DMS (2mM)	Smith 1987
Thiobacillus sp.	E7	2mM	*Sphagnum* sp. moss from a deodorisation unit	DMDS (2mM)	Smith 1987
Thiobacillus thioparus	E6	2mM	Pond water (Coventry, UK)	DMDS (2mM)	Smith & Kelly 1988
Hyphomicrobium sp.	I55	*N.D.*	Peat biofilter	DMS (1mM)	Zhang Y *et al*. 1991
Thiobacillus thioparus	DW44	*N.D.*	Peat biofilter	Thiosulfate (20mM)	Cho *et al*. 1991
Thiobacillus sp.	K4	*N.D.*	Biofilter	CS_2	Plas *et al*. 1991
Thiobacillus sp.	T5	1.3mM	Marine microbial mat (Texel, Netherlands)	Thiosulfate (10mM)	Visscher *et al.* 1991

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[1] "*Aerobacter aerogenes*".

[2] Isolation details of this strain do not appear in the literature.

Species	Strain	$[DMS]_{MAX}$	Isolated from	Isolation substrate (concentration)	Reference
Thiobacillus sp.	ANS-1	*N.D.*	Tidal sediment (Georgia, USA)	DMS (0.5mM)	Visscher & Taylor 1993a
Hyphomicrobium sp.	VS	1mM	Activated sludge	DMS (15μM)	Pol *et al*. 1994
Methylophaga sulfidovorans	RB-1	2.4mM	Marine microbial mat (Texel, Netherlands)	DMS (1.5mM)	de Zwart *et al*. 1996
Hyphomicrobium sp.	MS3	*N.D.*	Garden soil (Ghent, Belgium)	DMS/DMDS (1.4/1.1mM)	Smet *et al*. 1996
Xanthobacter tagetidis	TagT2C	2.5mM	*Tagetes patula* rhyzosphere	T2C(2.5mM)	Padden *et al*. 1997
Pseudonocardia asaccharolytica	580	*N.D.*	Animal rendering plant biofilter	DMDS (1mM)	Reichert *et al*. 1998
Pseudonocardia sulfidoxydans	592	*N.D.*	Animal rendering plant biofilter	DMS (0.5mM)	Reichert *et al*. 1998
Starkeya novella[3]	SRM	*N.D.*	Sewage (Kwangju, South Korea)	Thiosulfate (63mM)	Cha *et al*. 1999
Thiocapsa roseopersicina	M11	1mM	Marine microbial mat (Mellum, Germany)	Sulfide (1.6mM)	Jonkers *et al*. 1999
Methylobacterium podarium	FM1	*N.D.*	*Homo sapiens* foot	MMA (20mM)	Vohra 2000
Hyphomicrobium sulfonivorans	S1	*N.D.*	Garden soil (Warwickshire, UK)	$DMSO_2$ (10mM)	Borodina *et al*. 2002
Arthrobacter sulfonivorans	ALL/A	*N.D.*	*Allium aflatunense* rhizosphere	$DMSO_2$ (10mM)	Borodina *et al*. 2002
Arthrobacter sulfonivorans	ALL/B	*N.D.*	*Allium aflatunense* rhizosphere	$DMSO_2$ (10mM)	Borodina 2002

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[3] "*Thiobacillus novellus*".

Species	Strain	$[DMS]_{MAX}$	Isolated from	Isolation substrate (concentration)	Reference
Arthrobacter methylotrophus	TGA	*N.D.*	*Tagetes minuta* rhizosphere	$DMSO_2$ (10mM)	Borodina *et al*. 2002
Methylobacterium podarium	FM4	1mM	*Homo sapiens* foot	MMA (20mM)	Anesti *et al*. 2004
Hyphomicrobium sulfonivorans	CT	*N.D.*	*Homo sapiens* teeth	DMS (1mM)	Anesti *et al.* 2005
Hyphomicrobium sulfonivorans	DTg	*N.D.*	*Homo sapiens* tongue	DMS (1mM)	Anesti *et al.* 2005
Methylobacterium thiocyanatum	MM4	*N.D.*	*Homo sapiens* tongue	MMA (20mM)	Anesti *et al.* 2005
Methylobacterium extorquens	MM9	*N.D.*	*Homo sapiens* tongue	Methionine (5mM)	Anesti *et al.* 2005
Methylobacterium sp.	MM10	*N.D.*	*Homo sapiens* tongue	Cysteine (5mM)	Anesti *et al.* 2005
Micrococcus luteus	MM7	*N.D.*	*Homo sapiens* teeth	MMA (20mM)	Anesti *et al.* 2005
Bacillus licheniformis	3S(b)	*N.D.*	*Homo sapiens* gingivae	DMS (1mM)	Anesti *et al.* 2005
Bacillus licheniformis	2Tgb	*N.D.*	*Homo sapiens* tongue	DMS (1mM)	Anesti *et al.* 2005
Brevibacterium casei	3Tg	*N.D.*	*Homo sapiens* tongue	DMS (1mM)	Anesti *et al.* 2005
Brevibacterium casei	3S(a)	*N.D.*	*Homo sapiens* gingivae	DMS (1mM)	Anesti *et al.* 2005
Mycobacterium fluoranthenivorans	DSQ3	*N.D.*	River sediment (London, UK)	DMA (10mM)	Boden 2005, Boden *et al.* 2008
Methylophaga sp.	DMS001	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS002	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS003	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS004	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS007	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS009	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
"Methylophaga thiooxidans"[4]	DMS010	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007

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[4] "*Methylophaga* sp. DMS010".

Species	Strain	$[\mathrm{DMS}]_{\mathrm{MAX}}$	Isolated from	Isolation substrate (concentration)	Reference
Methylophaga sp.	DMS011	*N.D.*	*Emiliania huxleyi* culture	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS021	*N.D.*	Rock pool water (Coral Beach, UK)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS026	*N.D.*	Sea water (English channel)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS039	*N.D.*	Sea water (Achmelvich, UK)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS040	*N.D.*	Sea water (Achmelvich, UK)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS043	*N.D.*	Sea water (Achmelvich, UK)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS044	*N.D.*	Sea water (Achmelvich, UK)	DMS (50μM)	Schäfer 2007
Methylophaga sp.	DMS048	*N.D.*	Rock pool water (Coral Beach, UK)	Formate (10mM)	Schäfer 2007
Methylophaga aminisulfidivorans[5]	MP*	*N.D.*	Sea water (Mokpo, South Korea)	Methanol (220mM)	Kim HG *et al.* 2007
Sphingomonas melonis	ET35	*N.D,*	River sediment (London, UK)	Methanol (25mM)	Boden *et al.* 2008
Hyphomicrobium facile	-	*N.D.*	Marsh sediment (De Bruuk, Netherlands)	DMS (50μM)	Haaijer *et al.* (2008)
Microbacterium sp.	NTUT26	*N.D.*	Wastewater sludge from a wood pulp factory (Taiwan)	DMS (1.6mM)	Shu & Chen (2009)

Table 1.2. Known *Bacteria* capable of using DMS as a carbon source with details of isolation and maximal concentration of DMS for growth. (*N.D.* = Not determined; DMS = Dimethylsulfide; DMSO = Dimethylsulfoxide; $\mathrm{DMSO_2}$ = Dimethylsulfone; Thiometon = *O*,*O*-dimethyl phosphorodithioate; T2C = Thiophene-2-carboxylate; DMDS = Dimethyldisulfide; MMA = Monomethylamine).

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[5] "*Methylophaga aminosulfidovorans*"

Species	Strain	Product of DMS oxidation	Isolated from	Isolation substrate	Reference
Thiocystis sp.	A	DMSO	Salt Pond (MA, USA)	Sulfide	Zeyer *et al.* (1987)
Delftia acidovorans[6]	DMR-11	DMSO	Peat biofilter.	Peptone	Zhang L *et al.* (1991)
Methylomicrobium pelagicum	NI	DMSO	Sea water (Japan)	Methane	Fuse *et al.* (1998)
Sagittula stellata	E-37	DMSO	Pulp mill effluent	Kraft black liquor	Gonzalez *et al.* (1997)
Rhodovulum sulfidophilum	SH1	DMSO	Seawater	Bicarbonate	Hanlon *et al.* (1994)
Acinetobacter sp.	20B	DMSO	Soil (Japan)	Succinate (37mM)	Horinouchi *et al.* (1997)
Pseudomonas fluorescens	76	DMSO	Unknown	Unknown	Ito *et al.* (2007)
Thiocapsa roseopersicina	M1	DMSO	Marine microbial mat (Mellum, Germany)	Sulfide (1.6mM)	Visscher & van Gemerden (1991)
Glaciecola sp.	DMS049	*N.D.*	Sea water (English channel)	DMS (50μM)	Schäfer 2007
Marinobacter sp.	DMS050	*N.D.*	Sea water (English channel)	DMS (50μM)	Schäfer 2007
Marinobacter sp.	DMS054	*N.D.*	Sea water (English channel)	DMS (50μM)	Schäfer 2007

Table 1.3. *Bacteria* capable of oxidising DMS without assimilation of carbon or sulfur into biomass.

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[6] "*Pseudomonas acidovorans*".

1.3 Dimethylsulfoxide

Dimethylsulfoxide (DMSO) is a colourless, odourless liquid (MP = 18.5°C, BP = 189°C) which has a disputed degree of toxicity to *Eukarya* and *Bacteria* and *Archaea* (de la Torre *et al.* 1975; de la Torre 1981; Yellowless *et al.* 1980). Since its discovery by Saytzeff (1866), DMSO has found extensive use as a dipolar aprotic solvent in the chemical industry and in medicine and has thus been widespread in the Western world since the late 19th century. DMSO is also of medical interest as a drug delivery system and has been shown to facilitate the movement of various compounds across lipid membranes (Leake 1967). DMSO is metabolised by most mammals to $DMSO_2$, DMS or DMDS, which are then excreted in the urine and breath (Williams *et al.* 1966; Rammler & Zaffaroni 1967; Wood 1971). In nature, DMSO is found (amongst other sulfoxides) in various cruxiferous vegetables such as *Brassica oleracea* var. capitata L. (Cabbage), in some phytoplankton, such as *Emiliania huxleyi* (Kjær 1977) and in cereals such as *Hordeum vulgare* L. (Barley; Anness *et al.* 1979). DMSO reduction to DMS *in vitro* has been demonstrated to varying degrees in a diverse range of *Eukarya*, which are listed in Table 1.4. Given the wide range of *Eukarya* capable of reducing DMSO to DMS *in vitro*, it would be anticipated that many of these organisms are capable of DMS production *in situ* from DMSO present in soils.

Scientific name	Common name	Reference
Allium cepa L.	Onion	Smale *et al.* 1975
Arachis hypogaea L.	Peanut	Smale *et al.* 1975
Bos taurus L.	Cattle	Tiews et al. 1975
Cucumis sativus L.	Cucumber	Smale *et al.* 1975
Felix silvestris catus L.	Domestic cat	DiStefano & Borgstedt 1964
Fragaria vesca L.	Woodland strawberry	Smale *et al.* 1975
Glycine max L.	Soya bean	Smale *et al.* 1975
Helianthus annuus L.	Sunflower	Smale *et al.* 1975
Lactuca sativa L.	Lettuce	Smale *et al.* 1975
Malus domestica Borkh.	Apple	Smale *et al.* 1975
Phaseolus vulgaris L.	Common bean	Smale *et al.* 1975
Pisum sativum L.	Pea	Smale *et al.* 1975
Prunus persica L.	Peach	Smale *et al.* 1975
Pyrus communis L.	European pear	Smale *et al.* 1975
Saccharomyces cerevisiae	Brewers' yeast	Anness *et al.* 1979
Solanum tuberosum L.	Potato	Smale *et al.* 1975
Trifolium repens L.	White clover	Smale *et al.* 1975
Triticum aestivum L.	Common wheat	Smale *et al.* 1975
Zea mays L.	Maize	Smale *et al.* 1975

Table 1.4. *Eukarya* capable of reduction of DMSO to DMS *in vitro*.

The major environmental source of DMSO is through the photooxidation of DMS, particularly in the surface oceans and lower atmosphere (Lovelock *et al.* 1972). Light-independent chemical oxidation of DMS by peroxide radicals is also a source of DMSO in aquatic environments (Snow *et al.* 1976). Bacterial oxidation of DMS through the DMS dehydrogenase pathway in phototrophic *Bacteria* also provides a source of marine DMSO (Simó *et al.* 1998).

The first organism isolated into pure culture which was capable of using DMSO as a sole carbon, sulfur and energy source was *Hyphomicrobium* S (de Bont *et al.* 1981). Relatively few isolates have been cultured on DMSO, probably due to the varied opinions on the toxicity of the compound, which has caused most workers to use low concentrations (≤1mM) for culture work; however, it was been recently shown that, in *H. denitrificans*, low concentrations of DMSO are not usable as a carbon source, and that levels ≥20mM are optimal (Murakami-Nitta *et al.* 2002).

DMSO has been shown to act as a sole sulfur source for algae such as *Chlorella fusca* 211-8b (Krauss & Schmidt 1987).

DMSO can be used as a terminal electron acceptor by a wide range of *Bacteria*, ranging from *Gammaproteobacteria* such as *Escherichia coli*, *Proteus vulgaris* and *P. aeruginosa* to *Deltaproteobacteria* including *Desulfovibrio* spp. and *Epsilonproteobacteria* such as *Sulfurospirillum deleyianum* (previously "*Spirillium* sp. DL-1") (Ando *et al.* 1957; Zinder & Brock 1978b). The redox potential for the reduction of DMSO to DMS is +160mV (Smith 1987) – intermediate between those of the fumarate/succinate and nitrate/nitrite couples – indicating that the coupling of DMSO reduction to ATP synthesis by *E. coli* should be energetically favourable. Reduction of DMSO to DMS has been observed in *Rhodobacter capsulatus* (previously *Rhodopseudomonas capsulata*) with no apparent coupling to the respiratory chain (Yen & Mars 1976).

In *Bacteria* capable of using DMSO as a sole carbon and energy source, DMSO reductase catalyses the conversion of DMSO to DMS, which is then further dissimilated to formaldehyde *via* the DMS monooxygenase pathway.

1.4 Dimethylsulfone

Dimethylsulfone ($DMSO_2$) or “methylsulfonylmethane” is a non-toxic, crystalline solid (MP = 109°C, BP = 238°C) which is produced during the atmospheric oxidation of DMS (Watts *et al.* 1990). Although production of $DMSO_2$ has been demonstrated in the marine environment, methodological limitations have thus far limited its quantification (de Mora *et al.* 1996).

$DMSO_2$ is produced by various plants and animals, including *Equisetum arvense* L. (Horsetail), *Cladonia deformis* (Lesser sulfur-cup lichen), *Pinus palustris* L. (Longleaf pine) and most mammals (Burger *et al.* 2006; Kjær 1977; Merck 1989; Pfiffner & North 1940), presumably as a result of the detoxification of DMS which is, in turn, formed during the detoxification of MT.

$DMSO_2$ is a well-characterised sulfur-source for heterotrophic bacteria including *Pseudomonas putida* and *Escherichia coli* (reviewed by Kertesz 1999); however, relatively few organisms have been isolated which can use $DMSO_2$ as a carbon and energy source (Borodina 2002; Borodina *et al.* 2000, 2002). *Hyphomicrobium sulfonivorans* is, to date, the best characterised $DMSO_2$-oxidising bacterium, which has been shown to reduce $DMSO_2$ to DMSO (*via* $DMSO_2$ reductase), DMS and then MT, before assimilating the carbon *via* the serine cycle. The fate of sulfur in *H. sulfonivorans* has been demonstrated to be sulfate, a common end-product of metabolism of DMS, DMSO and $DMSO_2$ in almost all other strains characterised to date (Borodina *et al.* 2002). Although originally isolated from garden soil, strains of *H. sulfonivorans* have been isolated from environments as diverse as biofilters, freshwater lakes and the human mouth (Anesti *et al.* 2005; Dejonghe *et al.* 2003; Moosvi *et al.* 2005). $DMSO_2$ has found considerable use as a dietary supplement in the last 10 years (reviewed by Parcell 2002); although there is currently no evidence for any benefits of $DMSO_2$-consumption, it would be assumed that an increased level of $DMSO_2$ in the body would cause a shift in the methylotrophic populations of the natural flora.

The principal enzymes of bacterial $DMSO_2$ metabolism identified are $DMSO_2$ monooxygenase (Endoh *et al.* 2005) and $DMSO_2$ reductase (Borodina 2002; Borodina *et al.* 2002). The former is a reduced flavin mononucleotide ($FMNH_2$)-dependent monooxygenase which catalyses the oxidation of $DMSO_2$ to methanesulfonate (MSA) and formaldehyde prior to sulfur-assimilation in *P. putida*. The latter is, as yet, uncharacterised but has been demonstrated in terms of the reduced methyl viologen (MV)-dependent reductase activity in $DMSO_2$-grown *H. sulfonivorans* and *Arthrobacter* spp. cells (Borodina 2002). DMS is a common metabolic intermediate in all known pathways of $DMSO_2$ catabolism.

1.5 Thiosulfate and polythionates

1.5.1 Thiosulfate

Thiosulfate ($S_2O_3^{2-}$) is a sulfur oxy-anion comprising a sulfate (SO_4^{2-}) ion in which one of the oxygen atoms has been replaced by sulfur. The "outer" (sulfane) sulfur has an oxidation state of -2 whereas the "inner" (sulfonate) sulfur has an oxidation state of +6. Hydrolysis of thiosulfate can yield either elemental sulfur and sulfite or sulfide and sulfate; as such, the mechanisms of enzymatic thiosulfate oxidation are thought to share properties with those of sulfide and sulfur oxidation (Roy & Trudinger 1970). Thiosulfate is unstable in acidic solutions, the thiosulfuric acid ($H_2S_2O_3$) formed spontaneously decomposing to elemental sulfur and sulfur dioxide and, as such, thiosulfate has a low half-life in environments of low pH such as peatlands or in acid mine drainage. Although thiosulfate was not generally considered to be present in seawater, several studies have now confirmed its presence (along with that of polythionates) with concentrations ranging from 3μM (Wilmot & Vetter 1992) to around 300μM (Vetter *et al.* 1989; Ciglenečki & Ćosović 2005). It is worth noting that the low 3μM concentrations of thiosulfate were found in water populated by *Solemya reidi* Bernard (Gutless awningclam), which is known to contain thiosulfate-oxidising symbionts such as *Thiomicrospira* spp., which would cause a decrease in the local thiosulfate concentration (Kraus *et al.* 1992).

Thiosulfate is formed by the hydrolysis of elemental sulfur in hot, aqueous environments at all ranges of pH (Xu *et al.* 2000). In aquatic environments, particularly in the marine environment, thiosulfate is formed chemically from sulfide *via* elemental sulfur and sulfite (Kuenen 1975; Nedwell 1982):

$$2H_2S + O_2 \rightarrow 2S^0 + 2H_2O$$

$$H_2S + 2O_2 \rightarrow SO_3^{2-} + H_2O$$

$$SO_3^{2-} + S^0 \rightarrow S_2O_3^{2-}$$

This non-enzymatic condensation of elemental sulfur and sulfite to yield thiosulfate has been demonstrated in cell-free extracts both of *T. thioparus* and *Acidithiobacillus thiooxidans* (previously *T. thiooxidans*; Suzuki & Silver 1966).

1.5.2 Polythionates

The polythionates or sulfanedisulfonates (Schmidt 1957) are a homologous series of sulfur oxy-anions of the general formula $S_nO_6^{2-}$ where $n \geq 3$. Their structure consists of a chain of $n - 2$ sulfur atoms between two terminal SO_3^{2-} groups. The first reported polythionate to be synthesised and purified was tetrathionate ($S_4O_6^{2-}$) by Fordos and Gelis (1842), followed by trithionate ($S_3O_6^{2-}$) by Langlois (1866). The largest polythionate purified to date is hexacotathionate ($S_{24}O_6^{2-}$) (Mandalasi 2002), though compounds larger than hectatetracontathionate ($S_{140}O_6^{2-}$) are believed to exist (Weitz *et al.* 1956) and there is some evidence of their detection by chromatographic methods (Mandalasi 2002). The relative stability of the polythionates is generally considered to decrease as the chainlength increases. The exception to this is trithionate, which is particularly unstable, making tetrathionate the most stable polythionate. Geochemical formation of polythionates typically occurs due to the pyrite-catalysed oxidation of thiosulfate (Xu & Schoonen 1995). It has been suggested that mineral surfaces provide a catalyst for the oxidation of thiosulfate to tetrathionate, which can further combine with thiosulfate to form pentathionate and sulfite (Xu 1997):

$$S_2O_3^{2-} + S_4O_6^{2-} \rightarrow SO_3^{2-} + S_5O_6^{2-}$$

It is not known whether iron- or sulfide- oxidising bacteria associated with the mineral surfaces play a role in catalysing the formation of pentathionate. A second process for the formation of pentathionate from tetrathionate is also possible which yields trithionate (Xu 1997):

$$2S_4O_6^{2-} \rightarrow S_3O_6^{2-} + S_5O_6^{2-}$$

In acidic environments, trithionate hydrolyses rapidly to yield sulfate and thiosulfate (Meyer & Opsina 1982):

$$S_3O_6^{2-} + H_2O \rightarrow SO_4^{2-} + S_2O_3^{2-} + 2H^+$$

Microbial production of polythionates from thiosulfate is well documented in various species including autotrophs from the *Alphaproteobacteria* and *Betaproteobacteria* such as *Starkeya novella* (previously *T. novellus*) and *T. thioparus* and heterotrophs from the *Gammaproteobacteria* such as *Pseudomonas aeruginosa*, *P. stutzeri* (previously *Achromobacter stutzeri*), *P. fluorescens*, *Escherichia coli* (Starkey 1934) and *Pseudomonas* strain 1 (previously *Thiobacillus trautweinii*; Trautwein 1924). In addition to validated strains deposited in culture collections, a number of uncharacterised isolates have been found which can oxidise thiosulfate to polythionates (Trudinger 1967). Members of the *Deltaproteobacteria* such as *Desulfovibrio* spp. and *Desulfobulbus* spp. isolated from marine and freshwater sediments produce thiosulfate and trithionate during sulfate-reduction. Tetrathionate is also produced in low concentrations by such organisms but this is probably due to chemical oxidation of thiosulfate in the medium (Sass *et al.* 1992). Tetrathionate is produced from thiosulfate by a diverse range of heterotrophic *Gammaproteobacteria* and by *Eukarya* such as *Rhodotorula* spp. (Kurek 1985); however, it is only linked to energy metabolism in relatively few organisms, including *Archaea* such as *Natronorubrum* spp. and the thermophilic bacterium *Catenococcus thiocyclus* (Mason & Kelly 1988; Sorokin *et al.* 1996*a*; Sorokin 2003; Sorokin *et al.* 2005*a*; Vedenina & Sorokin 1992). It is worth noting that chemical oxidation of thiosulfate to tetrathionate has been observed in *Spirillum winogradskii* during heterotrophic growth and has been found to be caused by the presence of reactive oxygen species formed during aerobic growth, in a reaction analogous to those of the classical laboratory syntheses of polythionates (Willstätter 1903). Although an increase in growth yield (*Y*) is observed in *S. winogradskii* cultures grown on acetate in the presence of thiosulfate, since the enzymes of thiosulfate oxidation are absent and thiosulfate oxidation is not coupled to ATP synthesis, it is thought that thiosulfate increases the antioxidant capacity of the cells, leading to more efficient substrate utilisation, which, in turn, causes an increase in *Y* (Podkopaeva *et al.* 2005). It would be anticipated from the wide range of organisms (particularly *Eukarya*) which can oxidise DMS to DMSO that DMS may play a similar role in increasing cellular antioxidant capacity.

Thiosulfate oxidation to tetrathionate accompanied by an energy gain has been described in heterotrophic marine bacteria such as *Pseudomonas* 16B (previously "Marine pseudomonad 16B"; Tuttle & Jannasch 1972; 1973; Tuttle 1980; Tuttle *et al.* 1983), which shows some similarities to *Methylophaga* spp. and can make additional energy gain from the coupling of ATP synthesis to Mn^{2+} oxidation (Ehrlich & Salerno 1990).

Obligately heterotrophic sulfur-oxidising bacteria can be divided into two groups. The first are those which oxidise sulfur compounds incompletely to tetrathionate, which are widely distributed in marine environments and in soda lakes (Sorokin *et al.* 1996*b*; Tuttle & Jannasch 1972; 1973; Tuttle 1980; Tuttle *et al.* 1983) and these have been discussed in previous paragraphs. The second group are those which oxidise inorganic sulfur compounds completely to sulfate, of which relatively few have been described. *Dechlorimonas* sp. LMD 61.11 (Previously *Thiobacillus* Q; Gommers & Kuenen 1988), *Limnobacter thiooxidans* (Spring *et al.* 2001), *Ottowia thiooxydans* [*sic.*](Spring *et al.* 2004), *Tepidomonas ignava* and *Tepidomonas aquatica* (Moriera *et al.* 2000) are all members of the *Betaproteobacteria*; *Bosea thiooxidans* (Das *et al.* 1996), *Roseinatronobacter thiooxidans* (Sorokin *et al.* 2000), *Albidovulum inexpectatum* (Albuquerque *et al.* 2002), *Ruegeria pomeroyi* (González *et al.* 2003) and *Citreicella thiooxidans* (Sorokin *et al.* 2005*b*) are all members of the *Alphaproteobacteria*.

1.5.3 Metabolism

The oxidation of thiosulfate by chemolithotrophic bacteria is well documented and was originally demonstrated to be coupled to both carbon dioxide assimilation and to ATP synthesis in *Halothiobacillus neapolitanus* (Previously *Thiobacillus neapolitanus*; Kelly 1965; Kelly & Syrett 1963; 1964; 1966) and can be uncoupled using 2,4-dinitrophenol (DNP), which does not inhibit thiosulfate oxidation but prevents ATP synthesis. The oxidation of inorganic sulfur compounds (including thiosulfate and polythionates) by chemolithotrophic bacteria may be coupled to various types of phosphorylation to yield ATP (Peck 1960; London & Rittenberg 1964). Members of the *Aquificae* such as *Aquifex* spp., *Hydrogenobacter* spp. and *Hydrogenobaculum* spp. oxidise thiosulfate during hydrogen oxidation (Stöhr *et al.* 2001).

Metabolism of polythionates as an energy source for chemolithotrophs grown in chemostat culture has been studied in a variety of organisms, particularly *Thermithiobacillus tepidarius* (previously *Thiobacillus tepidarius*; Wood & Kelly 1985). Two pathways of inorganic sulfur compound oxidation in bacteria have been identified: the Kelly-Friedrich pathway (also known as the "*Paracoccus* sulfur oxidation" (PSO) pathway) and the tetrasulfur intermediate (S_4I) pathway.

A variety of marine isolates including *Hydrogenovibrio marinus* (Nishihara 2005), *Thiomicrospira* spp. and *Tetrathiobacter* spp. have been shown to use thiosulfate and polythionates as energy sources (Miyake *et al.* 2007).

1.5.3.1 The Kelly-Friedrich pathway

The Kelly-Friedrich pathway is found in *P. versutus*, *P. denitrificans*, *Xanthobacter* spp. and *S. novella* (Kelly *et al.* 1997) and proceeds without the formation of tetrathionate as an intermediate. A schematic of the Kelly-Friedrich pathway of thiosulfate oxidation is given in Figure 1.1. The Kelly-Friedrich pathway is the better-characterised of the two pathways of inorganic sulfur compound oxidation and is encoded by the *sox* gene cluster, which encodes the thiosulfate-oxidising multi-enzyme system (TOMES) and associated cytochromes. The individual subunits of the TOMES have been well studied at a biochemical and molecular biological level (Friedrich *et al.* 2005). It has recently been shown that the Kelly-Friedrich pathway is also present in *Methylobacterium* spp. for which thiosulfate can act as an energy source during mixotrophic growth on C_1 compounds (Anandham *et al.* 2007). Mixotrophic growth of methylotrophs during growth on C_1 compounds (with the Kelly-Friedrich pathway) has also been reported in *Hyphomicrobium* spp., *Arthrobacter* spp. and in *M. sulfidovorans* (Borodina *et al.* 2000; de Zwart *et al.* 1996). Whilst the *sox* gene cluster which encodes the Kelly-Friedrich pathway enzymes has been found in the genome of *M. extorquens* AM1 (Friedrich *et al.* 2001), it should be noted that there is no experimental evidence, to date, of mixotrophic growth of this strain.

1

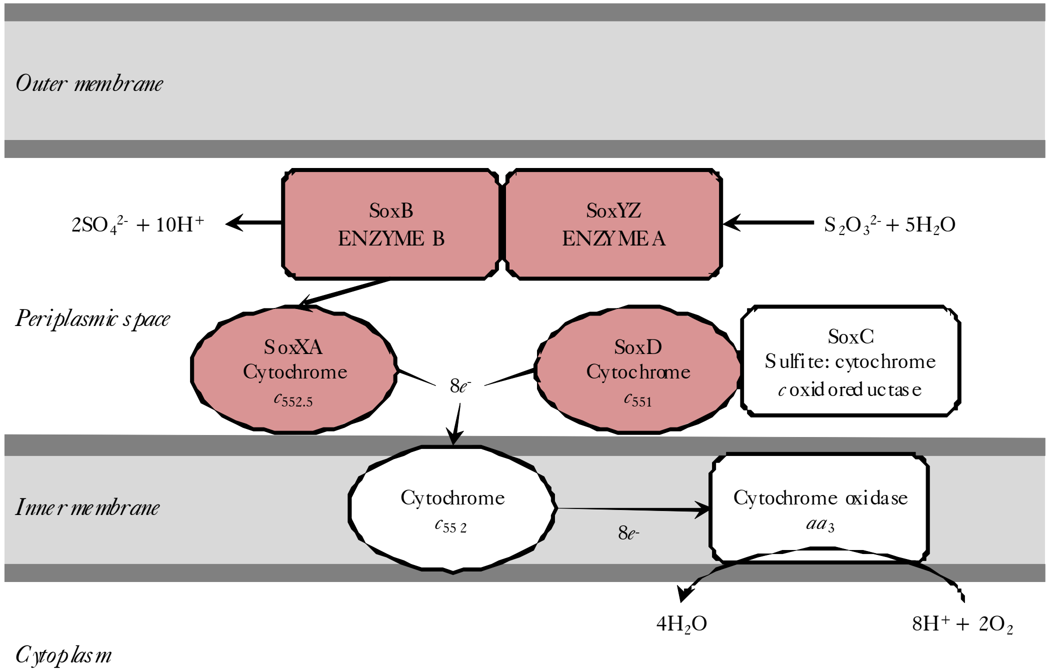

Figure 1.1. Simplified schematic representation of the Kelly-Friedrich pathway of thiosulfate oxidation in *Paracoccus versutus* (after Kelly 1988 and Kelly *et al.* 1997). Proteins represented in red indicate components of the TOMES.

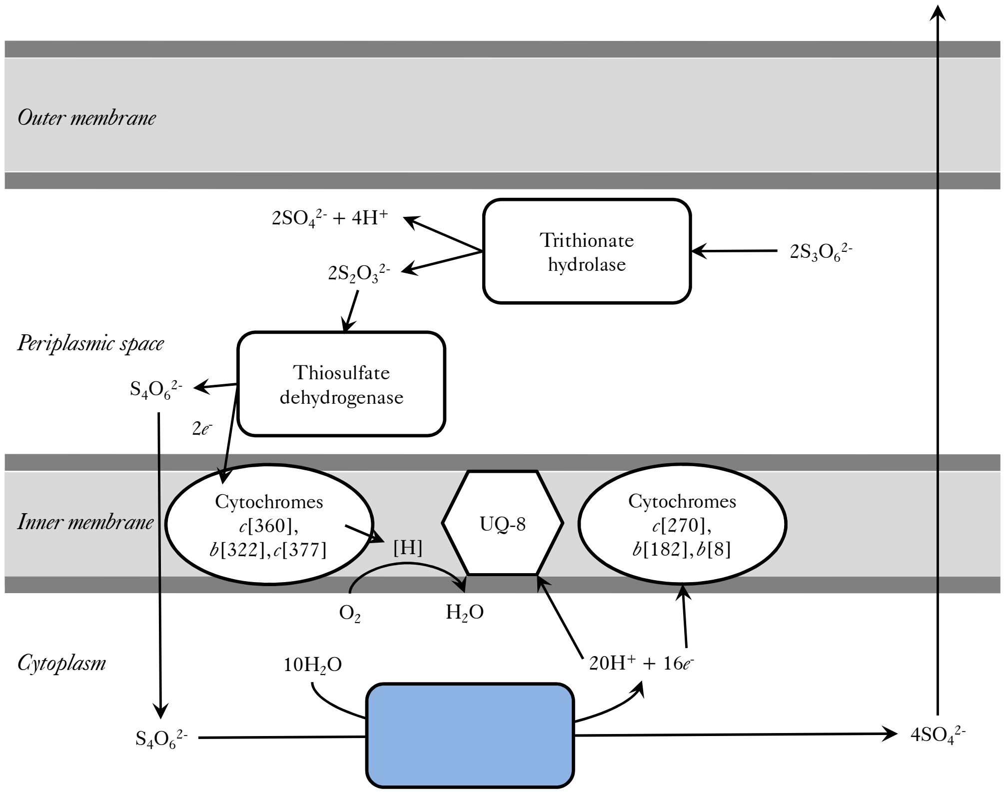

Figure 1.2. Simplified schematic representation of the S_4I pathway of sulfur oxidation in *Halothiobacillus neapolitanus* (after Kelly *et al.* 1997). Figures in square brackets indicate the mid-point potentials of cytochromes. The blue rectangle represents uncharacterised steps in the pathway, which are discussed in more detail in the text.

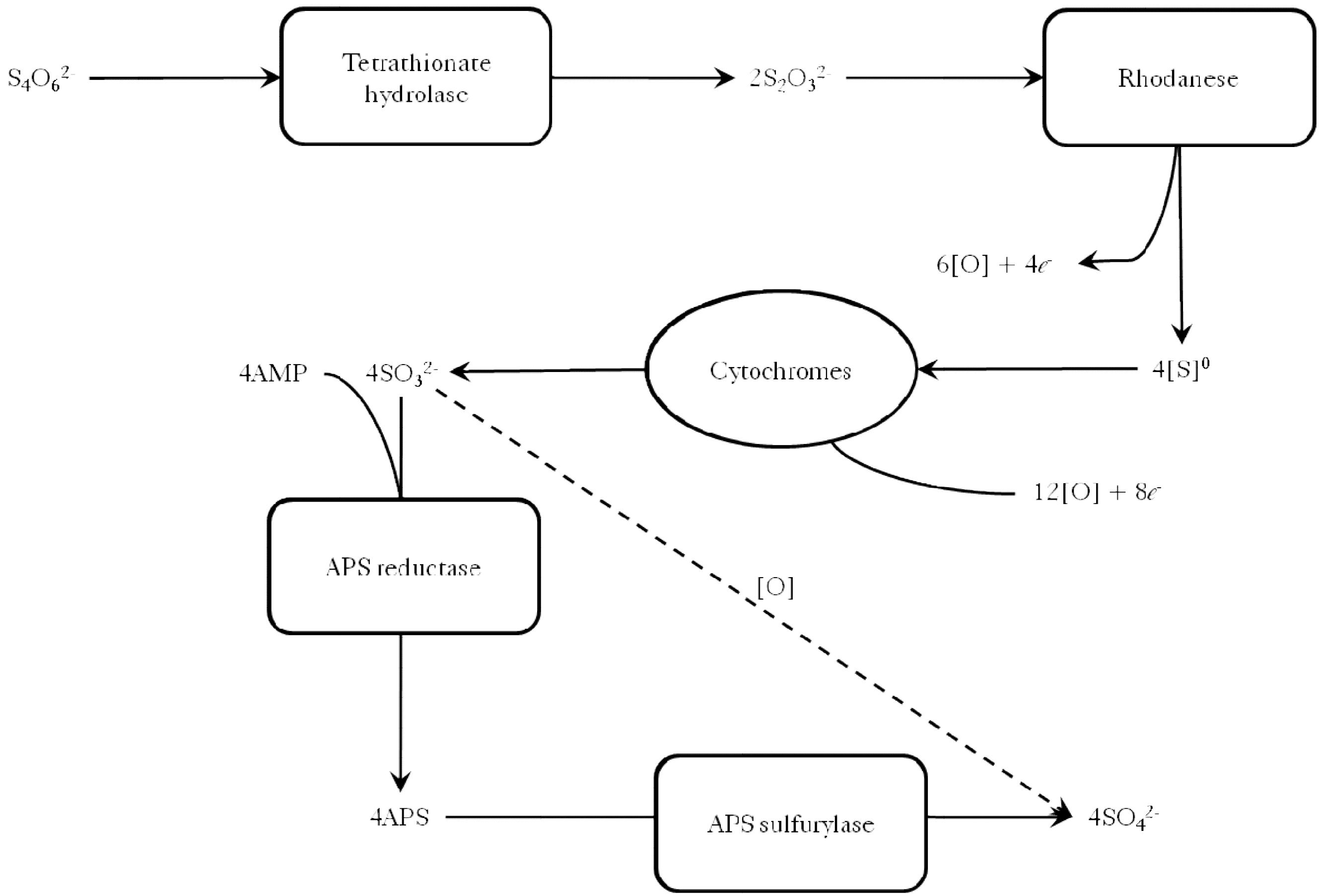

Figure 1.3. Simplified schematic representation of the sulfur-oxidation pathway in *Thiothrix ramosa* (after Odintsova *et al*. 1993).

1.5.3.2 The S_4I pathway

The S_4I pathway, found in *Thiobacillus* spp., *Acidithiobacillus* spp., *Halothiobacillus* spp., *Thermithiobacillus* spp., *Thiomonas intermedia* (previously *Thiobacillus intermedius*), *Thiomonas delicata* (previously *Thiobacillus delicates*) and *Tetrathiobacter* spp. (Kelly 1988; Sand *et al.* 1995; Dam *et al.* 2007), is less understood than the Kelly-Friedrich pathway, presumably due to the "difficult" nature of obtaining large amounts of biomass or establishing genetic systems in obligate autotrophs. A schematic representing the current knowledge of the S_4I pathway of thiosulfate and trithionate oxidation is given in Figure 1.2. The reactions indicated by the blue box in Figure 1.2 are currently unknown but various hypotheses are discussed in detail in Kelly *et al.* (1997). The most likely hypothesis would be tetrathionate metabolism *via* disulfane monosulfonate ($S_3O_3^{2-}$) linked back to trithionate metabolism at the beginning of the pathway (reviewed by Pronk *et al.*1990). Those organisms which oxidise thiosulfate to tetrathionate without further metabolism to sulfate could be considered to have an incomplete S_4I pathway. Data regarding the reactions between tetrathionate and cysteine indicate that cysteine residues present in enzymes would form cysteine-*S*-sulfonate and cysteine-*S*-thiosulfonate intermediates, prior to the formation of pentathionate and disulfane monosulfate, which would then be fed back into the pathway as previously described (Szczepkowiski 1958; Inglis & Liu 1970; Church & Evans 2008). The gene encoding an *o*-quinone-dependent tetrathionate hydrolase (*tetH*) has recently been cloned from *A. caldus* (previously *Thiobacillus caldus*; Rzhepishevska *et al.* 2007). The TetH protein catalyses the hydrolysis of tetrathionate to thiosulfate and pentathionate and is thought to represent the first of the "unknown" reactions indicated by the blue box in Figure 1.2, however, this is in contrast to the mechanism proposed by Kelly *et al.* (1997). A second gene (*doxD*) has been identified in *A. caldus*, which is thought to encode a thiosulfate:quinone oxidoreductase (Rzhepishevska *et al.* 2007). Genes similar to *doxD* have been cloned from members of the *Thermoprotei* such as *Acidianus ambivalens* and the *Gammaproteobacteria*, such as *A. ferrooxidans* (Müller *et al.* 2004).

Recently, complete thiosulfate oxidation to sulfate has been observed in *Enterobacter hormaechei*, which is described as being similar to that in *Thiomonas* spp., suggesting the presence of a complete S_4I pathway in a heterotrophic member of the *Gammaproteobacteria* (Kim *et al.* 2008).

1.5.3.3 Other pathways of inorganic sulfur compound metabolism

A third pathway of thiosulfate oxidation (Figure 1.3) has been identified in the facultatively chemolithotrophic member of the *Gammaproteobacteria*, *Thiothrix ramosa* (Odintsova *et al.* 1993) in which thiosulfate is oxidised to elemental sulfur by rhodanese; sulfur is then oxidised *via* a cytochrome-dependent mechanism to sulfite, which then reacts with adenosine 5'-monophosphate (AMP) *via* adenylyl sulfate reductase (APS reductase) to APS, which is finally oxidised to sulfate, *via* a putative sulfurylase. A similar pathway can be found in *Paracoccus pantotrophus* KL2 and KS1 (previously "*Thiobacillus* KL2" and "*Thiobacillus* KS1", Jordan *et al.* 1995) during growth on CS_2 and thiosulfate, in which thiosulfate is oxidised by rhodanese to elemental sulfur as with *Thx. ramosa*, which is then oxidised by a sulfur oxygenase to sulfite which is further oxidised to sulfate by the combined actions of sulfite dehydrogenase and chemical sulfite oxidation (Jordan *et al.* 1995, Jordan *et al.* 1997).

It is also worth briefly mentioning the role of rhodanese (thiosulfate:cyanide sulfurtransferase) in thiosulfate oxidation. Rhodanese catalyses the reaction between thiosulfate and cyanide to yield thiocyanate and sulfite in addition to the reaction between thiosulfate and α–lipoic acid to yield lipoate persulfide and sulfite (Silver & Kelly 1976). It should be noted that very high rhodanese activities (up to 1500nmol thiosulfate oxidised min^{-1} $(\text{mg protein})^{-1}$) have been found in pure samples of bovine hepatic catalase, indicating that the thiosulfate:cyanide sulfurtransferase reaction can occur in the absence of the true rhodanese enzyme (Lu 1983). There is some evidence that both the rhodanese and catalase enzymes exhibit rhodanese and catalase activity (DP Kelly, *unpublished data*).

1.6 Project aims

Three groups of DMS-oxidising *Bacteria* are of interest – those which assimilate DMS to biomass *via* the monooxygenase pathway; those which assimilate DMS to biomass *via* the methyltransferase pathway and those which oxidise DMS to DMSO without assimilation of DMS-carbon.

The major project aims are:

1. To purify and characterise the DMS monooxygenase from the model organism *Hyphomicrobium sulfonivorans*. Since *Hyphomicrobium* S is no longer available, it was necessary to select an alternative DMS-utilising *Hyphomicrobium* strain. *H. sulfonivorans* was chosen due to its growth on high concentrations of $DMSO_2$, giving high yields of biomass with DMS monooxygenase activity, suitable for protein purification.

2. To determine the pathway of DMS oxidation in "*Methylophaga thiooxidans*", which is thought to have the methyltransferase pathway. "*M. thiooxidans*" was selected since it was (at the time the work was performed) the only DMS-utilising member of the *Bacteria* to have a genome sequence available.

3. To investigate the bioenergetic effects of the oxidation of DMS to DMSO using *Sagittula stellata* as a model organism. *S. stellata* was selected since previous studies have confirmed its DMS-oxidising capabilites but the mechanism remained unknown. The genome sequence of *S. stellata* was publically available at the time the work was conducted.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

Unless otherwise stated, analytical grade chemicals were used throughout without further purification. Chemicals were provided by the Sigma-Aldrich Corporation (St Louis, MO, USA) unless otherwise stated. Dimethylsulfide was provided by Acros Organics (Geel, Belgium); absolute dimethylsulfoxide and flowers of sulfur were provided by Fluka Riedel-de-Haën. Sulfonates (with the exception of methanesulfonate) were provided by Lancaster Synthesis (Morecambe, UK).

Concentrated acids refer to 98% (*w/v*) sulfuric acid, 32% (*w/v*) hydrochloric acid and 78% (*w/v*) nitric acid. Glacial acetic acid refers to anhydrous acetic acid. "Molecular sieve" refers to crystalline sodium alumino-silicate molecular sieve type 13X in the form of 1.6mm pellets (British Drug Houses LTD, Poole, UK).

Reduced nicotinamide adenine dinuceotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purified of residual ethanol by dissolving in a small quantity of water and extracting ethanol in 5 volumes of diethyl ether before removing any remaining ether and water *in vacuo*. NAD(P)H was then dried over potassium hydroxide pellets at -20°C overnight before using to make stock solutions for enzyme assays, which were stored in the dark at -20°C.

Methanol (AristaR grade, British Drug Houses LTD, Poole, UK) was dried over molecular sieve and passed through a 0.44μM glass-fibre filter before use.

Analytical grade toluene and benzene (BDH) were washed with saturated magnesium chloride solution to remove impurities, partitioned and then dried over sodium sulfate before use.

Analytical grade carbon disulfide was washed with sulfuric acid, mercuric sulfate and mercury to remove contaminating DMS and carbonyl sulfide according to Skidmore (1979). Purified CS_2 was then dried over sodium sulfate prior to use in growth experiments.

Picric acid (supplied as aqueous slurry to prevent explosion) was cautiously dried between sheets of Whatman No. 2 filter paper before weighing in order to make solutions.

Buffers containing 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) were made at the working temperature in order to avoid changes in pH. The pH of Tris-containing buffers was determined using a compatible electrode.

2.1.1 Dimethylsulfide solutions

10mM stock solutions of DMS were prepared routinely either in sterile water (for use with non-marine isolates) or sterile marine ammonium mineral salts (MAMS) (for use with marine isolates). 73μL dimethylsulfide (presumed sterile) was added to 100mL aliquots of sterile MilliQ water (Millipore) or sterile MAMS in sterile 120mL serum vials sealed with butyl rubber bungs. Solutions were used within 6 months and concentrations checked periodically by gas chromatography, as outlined in 2.3.

2.1.2 Thiol solutions

In order to avoid auto-oxidation to disulfides, thiol solutions were prepared in sterile water which had been degassed *in vacuo* for 1 hour and regassed with nitrogen for 30 minutes. 20mL aliquots of prepared water were placed in 25mL glass serum vials and were saturated with the appropriate thiol. In the case of methanethiol (MT), the gas was bubbled through the solution for 15 minutes which produced a solution of approximately 0.3M MT (Smith 1988). All thiol solutions were diluted immediately before use into nitrogen-flushed water and the actual thiol concentration determined by gas chromatography, as outlined in Section 2.3 (Suylen & Kuenen 1986). In order to reduce adsorption, glassware used to contain thiols was coated with dimethyldichlorosilane before use and bungs were coated with PTFE.

2.1.3 Substituted-thiophene solutions

Solutions of thiophene-2-carboxylate (T2C), thiophene-3-carboxylate (T3C), thiophene-2-acetate (T2A) and thiophene-3-acetate (T3A) were prepared by dissolving an appropriate amount of the free acid in hot deionised water and neutralising with 5M sodium hydroxide solution before diluting to volume with further hot deionised water. Solutions were autoclaved before use. Thiophene, thiophene-2-methylamine (T2MA) and dibenzothiophene (DBT) were presumed to be sterile and were added directly to medium before use.

2.1.4 Formaldehyde solutions

Methanol-free formaldehyde solutions were prepared for use during growth experiments and enzyme assays by the thermal depolymerisation of paraformaldehyde, as previously described (Chongcharoen *et al.* 2005). For some assays, a commercially available 16% (*w/v*) methanol-free formaldehyde solution was used (Pierce Biotechnology Inc., Rockford, IL, USA).

2.1.5 Synthesis of polythionates

Sodium polythionates were synthesised and purified using modifications of methods for the synthesis of potassium polythionates. Salts were stored under argon in a desiccator at -20°C until required. Solutions were prepared immediately before use and were sterilised by filtration through polyvinylidene difluoride (PVDF) membranes of 0.22μm pore size. Headspaces of bottles containing polythionates were flushed with argon to prevent oxidation. Purity was assessed by methods outlined in Section 2.3.

Full details of synthetic methods are given in the Appendix.

2.1.5.1 Sodium trithionate

Sodium trithionate was synthesised using a modification of the methods of Willstätter (1903) and Wood & Kelly (1986).

2.1.5.2 Sodium tetrathionate

Sodium tetrathionate was prepared by the oxidation of thiosulfate using iodine, based on a modification (Wood *et al.* 1987) of the method of Trudinger (1961).

2.1.5.3 Sodium pentathionate

Sodium pentathionate was prepared using a method adapted from those of Kelly & Wood (1994) and Stamm *et al.* (1941) for synthesis of potassium pentathionate.

2.1.5.4 Sodium hexathionate

Sodium hexathionate was prepared using a modification of the method of potassium hexathionate synthesis by Weitz & Achterberg (1928).

2.1.5.5 Sodium heptathionate

Sodium heptathionate was prepared using the method for the synthesis of potassium heptathionate described by Wood & Kelly (1986).

2.1.6 Preparation of elemental sulfur solutions

For use in enzyme assays and growth experiments, four forms of elemental sulfur were employed in order to try to eliminate previously cited problems of the sulfur not being in a biologically available form for the organism or enzyme in question (LeFaou *et al.* 1990).

2.1.6.1 Flowers of sulfur

Flowers of sulfur (Sigma) were ground into a fine powder followed by tyndallisation at 105°C for 20 minutes followed by incubation at 30°C for 24 hours. A total of 3 cycles of tyndallisation were used to obtain sterile flowers of sulfur suitable for enzyme and growth experiments.

2.1.6.2 *Lac sulfuris*

100mL 15mM solution of flowers of sulfur in DMSO was poured into an equal volume of sterile water and precipitated sulfur was allowed to settle overnight. The supernate was dialysed against a large volume of water to remove DMSO and the resultant white, hydrophilic sol ("milk of sulfur" or "*lac sulfuris*") was stored in aliquots at 4°C until required. *Lac sulfuris* prepared in this way was found to contain approximately 1.1mmol mL^{-1} elemental sulfur, as assayed by method given in Section 2.3.3.

2.1.6.3 Wetted sulfur

40g finely ground flowers of sulfur were stirred vigorously with 60mL 0.05% (*v/v*) (*x*)-sorbitan mono-9-octadecanoate poly(oxy-1,2-ethanediyl) (Tween® 80) immediately prior to use in order to form a hydrophilic suspension. In enzyme assays where wetted sulfur was used, a negative control comprising 0.03% (*v/v*) Tween® 80 in water was used (Suzuki *et al.* 1999).

2.1.6.4 “Biologically active” sulfur

Prepared according to Fauque (1994) by mixing 1.5mL 0.6M sodium sulfite solution with 50mL 0.5M sodium sulfide solution and adding 8mL 2.7M sulfuric acid with rapid stirring. 48.5mL 0.6M sodium sulfite solution in 1.1M sulfuric acid was then added and the solution allowed to stand for 1 hour before being centrifuged at 22,000 × *g* for 15 minutes at 4°C. Sulfur was washed three times with 0.2M sodium chloride solution and resuspended in 30mL MilliQ water.

2.1.7 Synthesis of [$^{13}C_2$]-dimethylsulfide

Labelled DMS for stable-isotope probing experiments was prepared by a method adapted from that for labelled DMSO synthesis (Beerli & Borschberg 1991). 6.5g sodium sulfide nonohydrate was dissolved in 6.5mL sterile deionised water in a glass test-tube and cooled to 0°C in an ice-water bath with vigorous stirring. 5g [^{13}C]-methyl iodide (Cambridge Isotope Laboratories LTD, Andover, MA) was added dropwise over a period of 30 minutes then the reaction mixture incubated at 0°C for 5 hours, with stirring. 5mL of each 2M sodium hydroxide solution and 2M sodium thiosulfate solution were then added to destroy remaining methyl iodide. The reaction vessel was connected to a receiving bulb held at -170°C in liquid nitrogen and the reaction mixture allowed to warm to 40°C in a water bath. [$^{13}C_2$]-DMS was distilled from the reaction mixture for 90 minutes and then re-distilled into a sterile receiving bulb for 1 hour. Sterile deionised water was added to the receiving vessel to dissolve the [$^{13}C_2$]-DMS and the resulting solution transferred, with washings, to a sterile 1L serum vial which was then sealed with a butyl rubber bung. The concentration and purity of the [$^{13}C_2$]-DMS solution was assessed by gas chromatography as outlined in 2.3.2.1. 250mL of a 7mM solution of pure [$^{13}C_2$]-DMS was typically obtained.

2.1.8 Synthesis of cyclopropanol

Cyclopropanol for inhibition of methanol dehydrogenase was synthesised by biotransformation of cyclopropyl methyl ketone by *Rhodococcus erythropolis* DSM1069 using a method adapted from that of Overbeeke *et al.* (2003).

2.1.8.1 Growth of *Rhodococcus erythropolis* DSM 1069

R. erythropolis DSM1069 was growth in batch culture in a Fermac 300 Series fermenter (Electrolab LTD, Tewksbury, UK) in a 2L vessel at 30°C, pH 7.2 and was stirred at 250rpm and sparged with air at 3Lmin^{-1} for 72 hours in CBS medium supplemented with 0.05% (*w/v*) yeast extract and 0.5M cyclohexanol (Overbeeke *et al.* 2003). Inoculum (200mL) consisted of cells washed from slopes of an *R. erythropolis* DSM 1069 culture maintained on GYB medium pre-grown overnight on GYB broth.

2.1.8.2 Biotransformation of cyclopropyl methyl ketone

Cells were harvested from the culture medium at 14,000 × *g* at 4°C for 40 minutes before washing and resuspending in 50mM Tris-HCl pH 7.5 to a final volume of 500mL. 2.5mL cyclopropyl methyl ketone was added and the cells incubated in a sealed 2L Erlenmeyer flask at 25°C for 48 hours with gentle agitation.

2.1.8.3 Purification of cyclopropanol

The reaction mixture was saturated with sodium chloride and extracted in 100mL volumes of ether (1L in total). Combined extracts were dried over sodium sulfate and the ether was removed *in vacuo* at 30°C. Cyclopropanol was distilled at atmospheric pressure as previously described (Jongejan & Duine 1987).

2.1.9 Organisms

A list of bacterial strains used in this study (and their sources) is given in Table 2.1. All strains were maintained on their appropriate medium solidified with the addition of 2% (*w/v*) Noble agar (Difco). For long-term storage (≥ 2 weeks), strains were stored at -80°C in their growth medium with the addition of 8% (*v/v*) DMSO or 10% (*v/v*) glycerol.

Xanthobacter tagetidis TagT2C was grown on 2.5mM T2C with as previously described (Padden *et al.* 1997) with the addition of 0.4% (*w/v*) glycine at late exponential phase to facilitate rupturing of the cells at the French press (Wiegel 2006). *Paracoccus* spp. and *Methylobacterium* spp. were grown on CBS medium supplemented with 20mM thiosulfate or 25mM methanol, respectively. *Thiobacillus thioparus* Tk-m was grown on CBS medium supplemented with 2mM DMS or 20mM thiosulfate. *Methylophaga* spp. were maintained on MAMS medium supplemented with 25mM methanol. *Hyphomicrobium* sp. VS was grown on 2mM DMS as previously described (Pol *et al.* 1994). *Thiocapsa roseopersicina* M11 was grown on the acetate-citrate-fructose/sulfide-thiosulfate medium described by Malik (1983). Methane-grown cells of *Methylosinus trichosporium* OB3b were supplied as a frozen paste (-80°C) by Dr E. Borodina (University of Warwick). *Rhodococcus erythropolis* was grown on GYB medium. Marine heterotrophs were maintained on marine agar or marine broth (Difco).

Organism	Strain	Source	Reference
Hyphomicrobium sp.	VS	H. J. M. Op den Camp*	Pol *et al*. 1994
Hyphomicrobium sulfonivorans	S1^{T}	A. P. Wood†	Borodina *et al*. 2002
Methylobacterium podarium	FM4^{T}	A. P. Wood	Anesti *et al*. 2006
Methylobacterium thiocyanatum	ALL-SCN-P^{T}	A. P. Wood	Wood *et al*. 1999
Methylophaga aminisulfidivorans	MP^{T}	Y. A. Trotsenko‡	Kim HG *et al*. 2007
Methylophaga sulfidovorans	RB-1^{T}	DSMZ	de Zwart *et al*. 1998
"*Methylophaga thiooxidans*"	DMS010^{T}	H. Schäfer§	Schäfer 2007
Methylosinus trichosporium	OB3b	E. Borodina¶	Whittenbury *et al*. 1970
Paracoccus pantotrophus[7]	KL1	A. P. Wood	Jordan *et al*. 1997
Paracoccus versutus	A2^{T}	A. P. Wood	Taylor & Hoare 1969
Rhodococcus erythropolis	DSM1069	DSMZ	Overbeeke *et al*. 2003
Sagittula stellata	E-37^{T}	DSMZ	Gonazalez *et al*. 1997
Thiobacillus thioparus	Tk-m	DSMZ	Kanagawa & Kelly 1986
Thiocapsa roseopersicina	M11	H. Jonkers\\	Jonkers *et al*. 1999
Xanthobacter tagetidis	TagT2C^{T}	A. P. Wood	Padden *et al*. 1997

Table 2.1. Bacterial strains used in this study along with their sources.

* Radboud University, Nijmegen, Netherlands; † Dental Institute, King's College London, London, UK; ‡ Russian Academy of Sciences, Pushchino, Russia; § Warwick Horticultural Research International, Wellesbourne, UK; ¶ University of Warwick, Coventry, UK; \\ Rijksuniversiteit, Groningen, Netherlands; DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

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[7] "*Paracoccus denitrificans* KL1"

2.2 Growth of *Bacteria* in batch and continuous culture

2.2.1 Culture media

All solid media were solidified by the addition of 1.5% (*w/v*) Noble agar (Difco) prior to autoclaving. Phosphate salts were routinely autoclaved separately in 20% of the final medium volume and were added to the complete medium once cool.

2.2.1.1 Marine ammonium mineral salts (MAMS)

Marine ammonium mineral salts (MAMS) medium was adapted from Goodwin *et al.* (2001) and Thompson *et al.* (1995).

Per litre:

Sodium chloride	20.0g
Ammonium sulfate	1.0g
Calcium chloride dihydrate	0.2g
Magnesium sulfate heptahydrate	1.0g
Ferrous sulfate heptahydrate	0.002g
Sodium tungstate	0.003g
Sodium molybdate dihydrate	0.02g
Potassium dihydrogen phosphate	0.36g
Dipotassium hydrogen phosphate	2.34g
Trace elements solution "SL-10"	1.0mL
Vitamins solution "V10"	1.0mL

2.2.1.2 Nitrogen-free MAMS (N-MAMS)

Per litre:

Sodium chloride	20.0g
Calcium chloride dihydrate	0.2g
Magnesium sulfate heptahydrate	1.0g
Ferrous sulfate heptahydrate	0.002g
Sodium tungstate	0.003g
Sodium molybdate dihydrate	0.02g
Potassium dihydrogen phosphate	0.36g
Dipotassium hydrogen phosphate	2.34g
Trace elements solution "SL-10"	1.0mL
Vitamins solution "V10"	1.0mL

2.2.1.3 Complete basal salts (CBS)

Complete basal salts (CBS) medium was based on a modification (Wood & Kelly 1977, Boden *et al.* 2008) of the medium originally described by Taylor & Hoare (1969) for use with *Paracoccus versutus* ("*Thiobacillus* A2").

Per litre:

Ammonium chloride	0.2g
Magnesium sulfate heptahydrate	0.1g
Potassium dihydrogen phosphate	1.5g
Disodium hydrogen phosphate	6.3g
Trace elements solution "T"	10.0mL
Vitamins solution "V10"	1.0mL

2.2.1.4 Glucose-yeast-beef medium (GYB)

Per litre:

Glucose	4.0g
Yeast extract	4.0g
Beef extract	10.0g

2.2.1.5 Trace metals solution SL-10 for MAMS medium

Widdel *et al.* 1983.

Per litre:

Concentrated hydrochloric acid	5mL
Ferrous chloride tetrahydrate	1.5g
Zinc chloride	0.07g
Manganous chloride tetrahydrate	0.1g
Boric acid	0.006g
Cobaltous chloride hexahydrate	0.19g
Cupric chloride dihydrate	0.002g
Nickel chloride hexahydrate	0.024g
Sodium molybdate dihydrate	0.036g

2.2.1.6 Trace metals solution T” for CBS medium

Tuovinen & Kelly 1973.

Per litre:

Disodium EDTAate	50.0g
Sodium hydroxide	11.0g
Zinc sulfate hexahydrate	11.0g
Calcium chloride dihydrate	7.34g
Manganous chloride pentahydrate	2.50g
Cobaltous chloride hexahydrate	0.50g
Ferrous sulfate heptahydrate	7.50g
Cupric sulfate pentahydrate	0.20g
Ammonium molybdate pentahydrate	0.50g

2.2.1.7 Vitamin solution "V10"

Solution V10 (Heijthuijsen & Hansen 1986) was sterilised by filtration through a PVDF membrane of 0.22μm pore size and was stored in the dark at 4°C until required.

Per litre:

p-aminobenzoic acid	0.30g
Biotin	0.02g
Nicotinic acid	0.20g
Calcium pantothenate	0.10g
Pyridoxine dihydrochloride	0.50g
Lipoic acid	0.05g
Folic acid	0.05g
Cyanocobalamin	0.10g
Thiamine	0.20g
Riboflavin	0.10g

2.2.2 Growth in batch cultures

Batch cultures on DMS were routinely grown in 50mL volumes in 250mL Erlenmeyer flasks sealed with red rubber vaccine stoppers. Batch cultures on non-volatile substrates were grown in 250mL Erlenmeyer flasks with foam bungs.

Fed-batch fermenters were used for the cultivation of *T. roseopersicina* M11 under anaerobic conditions. A 4L culture volume in an LH Series 200 fermenter (LH Engineering LTD, Stoke Poges, UK) was maintained at 30 ± 1°C, stirred at 250rpm with two six-bladed Rushton impellors and gassed with sterile nitrogen at 2L min^{-1}. A light source was provided by a 60W tungsten filament lamp held at 30cm from the culture vessel. The *Chromataceae* medium of Malik (1983) was used with the addition of 200μM resazurin, to indicate the presence or absence of oxygen in the culture. The growing culture was monitored at 24 hour intervals by the qualitative determination of sulfide with plumbous acetate[8]. Once sulfide in the culture was depleted, it was "fed" as using the "neutral-sulfide solution" and "feed solution" of Malik (1983). Cultures were harvested at an OD_{440} of approximately 18.5.

2.2.3 Continuous cultures

All vessels used in continuous culture were coated with dimethyldichlorosilane (applied as a 0.15M solution in octamethylcyclotetrasiloxane) in order to minimise wall growth. Media for continuous culture were supplemented with 0.002% (*v/v*) Antifoam 289 (Sigma) prior to autoclaving in order to minimise foaming. Influent

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[8] Briefly, 0.5mL culture supernate was mixed with 0.5mL 1.6M plumbous acetate solution in a glass Bijou tube. The immediate formation of a black or grey precipitate was taken to be a crude indication of the presence of sulfide in solution.

compressed air was washed and saturated with water by passage through a Dreschel bottle filled with MilliQ water and 4mm glass beads before sterilising by passage through a HEPA-CAP™ filter capsule (Whatman PLC, Maidstone, UK). In order to prevent bacterial growth blocking the air-sparge arm, a Bunsen valve of silicone tubing was fitted over the arm. Acid and base for automatic titration was supplied from 500mL graduated burettes in order that the exact volume added over time could be monitored (Justin 1978). Effluent gases were dried through a 1L mixed bed of silica gel and molecular sieve before passage through a gas analyser to monitor effluent oxygen and carbon dioxide concentrations.

2.2.3.1 *Hyphomicrobium sulfonivorans* S1

H. sulfonivorans S1 (Borodina *et al.* 2000) was grown in continuous culture in 4L volume in an LH Series 200 fermenter (LH Engineering LTD), maintained at 30 ± 1°C, stirred at 300rpm and aerated with sterile air at 2L min^{-1}. CBS medium was used, supplemented with 40mM DMSO$_2$ as the growth-limiting substrate (D = 0.03h^{-1}). pH was controlled by automatic titration with 5M sodium hydroxide solution to maintain a pH of 7.4 ± 0.1.

2.2.3.2 "*Methylophaga thiooxidans*" DMS010

"*M. thiooxidans*" DMS010 was grown in continuous culture in a 1L or 2L volume in a Fermac 300 Series fermenter (Electrolab), maintained at 25 ± 1°C, stirred at 250rpm with two six-bladed Rushton impellors and aerated with sterile compressed air at 2L min^{-1}. MAMS was used, supplemented with either 30mM methanol or 15mM DMS as the growth-limiting substrate. pH was controlled by automatic titration with 4M sodium hydroxide solution and 2M sulfuric acid to maintain a pH of 7.2 ± 0.1.For comparative proteomics experiments, substrate concentration was normalised to give equivalent carbon concentrations: 30mM methanol, 15mM DMS or 6mM T3C.

For growth on DMS in continuous culture, various modifications were made to the fermentation system, based on those of Smith (1988) for growth of *Bacteria* on dimethyldisulfide (DMDS). Viton tubing (Watson-Marlow Bredel Pumps LTD, Falmouth, UK) was used for all connections between reservoirs and the vessel in order to prevent loss of DMS. An argon-filled balloon was attached to the air inlet filter of the medium reservoir to prevent loss or oxidation of substrate and to allow for decrease in volume of the residual medium. DMS-containing medium entered the culture through the air-sparge arm in order to ensure instant mixing and to prevent loss of substrate from the culture. Effluent gas was passed through a Carbon Cap™ filter capsule (Whatman) to remove any residual volatile compounds during the early stages of growth, prior to a steady-state being reached.

Silicone septa were positioned in the top-plate of each fermentation vessel to allow sampling of headspace gas. Septa were also present in the stoppers of medium reservoirs (20L glass carboys fitted with PTFE-coated silicone bungs) in order to allow monitoring of substrate concentration, in the case of volatile substrates.

Where secondary substrates were used (*e.g.* thiosulfate), these were metered separately into the culture vessel from 10× stock solutions at 1/10th of the medium flow rate in order to give an appropriate final concentration.

2.2.3.3 *Sagittula stellata* E-37

S. stellata E-37 was grown in continuous culture in a 2L volume in a Fermac 300 Series fermenter (Electrolab, UK), maintained at 30 ± 1°C, stirred at 350rpm with 2 six-bladed Rushton impellors and aerated with sterile compressed air at 3Lmin^{-1}. MAMS was used, supplemented with 12mM fructose or 2mM sodium succinate as the growth-limiting substrate. pH was controlled by automatic titration with 2M sodium hydroxide solution and 1M sulfuric acid to maintain a pH of 7.2 ± 0.1.

Where DMS was used as a secondary substrate, it was metered separately into the culture vessel from a 10× stock solution in MAMS at 1/10th of the medium flow rate in order to give an appropriate final concentration. DMS stock solutions were held under nitrogen in 1L glass aspirator bottles sealed with PTFE-coated silicone bungs. A nitrogen-filled balloon was attached to the air inlet filter to prevent loss or oxidation of the DMS and to allow for decrease in volume of the solution. DMS-containing solutions were fed into the vessel using Viton tubing and entered *via* the air-sparge arm, as described in Section 2.2.4.2.

2.2.4 Harvesting, washing and storage of cells

Cells were routinely harvested from cultures by centrifugation at 13,000 × *g* for 30 minutes at 4°C. Cells were washed and resuspended in dilution buffer (50mM

PIPES-HCl pH 7.4 containing 0.4M sodium chloride, 50mM magnesium sulfate and 0.01% (*w/v*) piscine gelatin). For organisms grown anaerobically, DTT was added to the dilution buffer to a final concentration of 50mM and the buffer sparged with argon before use (Gerhardt 1981). Cells were snap-frozen in liquid nitrogen and stored routinely at -80°C.

2.3 Analytical methods and techniques

Unless otherwise stated, all analytical determinations were carried out using appropriate volumetric glassware at 20°C in a waterbath. Glassware was cleaned between assays by soaking in chromic acid for 1 hour, followed by washing 4 times in MilliQ water, once with 50mM sodium hydroxide solution, once with 1M disodium EDTAate solution and 4 times with MilliQ water before drying in the oven. Glassware was cooled to 20°C in a waterbath before use. For ATP-determinations, glassware was soaked for 24 hours in sodium hypochlorite solution (≥8% available chlorine) before washing in MilliQ water and ethanol, baking in an oven at 120°C for 1 hour and cooling before use.

Unless otherwise stated, all spectrophotometric assays were conducted in seven-fold replicate in 1cm path length 1mL polymethacrylate (>320nm) cuvettes or 1cm path length 1mL quartz (<320nm) cuvettes.

2.3.1 Estimation of biomass

Growth of culture was routinely monitored by measuring optical density at 440nm (OD_{440}) in a DU-70 Spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA). Samples from chemostat cultures were used to prepare calibration curves relative to OD_{440} of dry weight of biomass (mg mL^{-1}). Cells were harvested by centrifugation at 13,000 × *g* for 30 minutes at 4°C and were resuspended to various OD_{440} values in sterile dilution buffer (0.4M sodium chloride, 50mM magnesium sulfate, 50mM PIPES-HCl pH 7.4, 0.01% (*w/v*) piscine gelatine; Gerhardt 1981). 1mL aliquots were dried to constant weight at 105°C in thin-walled glass vials. In the case of *H. sulfonivorans*, the relationship of dry weight being equal to 230mgmL^{-1} at an OD_{440} of 1.0 was used (E. Borodina, *personal communication*).

2.3.2 Quantification of organic compounds

2.3.2.1 Volatile organosulfur compounds and other volatiles

Organic compounds in culture headspaces were quantified by gas chromatography. 100µL volumes of culture headspace were injected into a GCD gas chromatograph (PYE Unicam Ltd., Cambridge, UK) fitted with a 4mm × 1m glass column packed with Poropak Q 80-100 mesh (Phase Separations LTD, Deeside, UK) at 200°C with nitrogen at 30mLmin^{-1} as the carrier gas. A flame ionization detector was used to detect compounds, and peak areas were integrated with a 3390A Integrator (Hewlett Packard, Berkshire, UK). For quantitative work, concentrations were calculated by regression analysis based on calibration curves constructed using standard solutions in an appropriate buffer or basal salts. Retention times for all compounds of interest were determined relative to that of *n*-hexane and are given in Table 2.2. The retention time of *n*-hexane was approximately 4 minutes.

Compound	M_W (Da)	Relative retention time
Methanethiol	48.11	0.305
Ethanethiol	62.13	0.509
Methanol	32.04	0.318
Ethanol	46.07	0.328
Carbon disulfide	76.10	0.537
Carbonyl sulfide	60.07	0.515
Dimethylsulfide	62.13	0.525
n-Hexane	86.18	1.000
Ethylmethylsulfide	76.16	1.015
n-Propanethiol	76.16	1.120
sec-Propanethiol	76.16	1.194
Thiophene	84.14	1.320
Ethylvinylsulfide	88.17	1.670
n-Butanethiol	90.19	1.702
sec-Butanethiol	90.19	1.726
Diethylsulfide	90.19	1.804
Allylmethylsulfide	88.17	1.808
Dimethyldisulfide	94.20	2.217
Allyl isothiocyanate	99.15	4.826
Dipropylsulfide	118.24	6.747
Thioanisole	124.20	16.710
Dibutylsulfide	146.29	26.730

Table 2.2 Retention times relative to *n*-hexane for various volatile compounds measured by GC using a Poropak Q 80-100 mesh column at 200°C. The retention time of *n*-hexane was approximately 4 minutes.

2.3.2.2 Dimethylsulfoxide

DMSO was quantified in solution by reduction to DMS, which was subsequently measured as outlined in 2.3.2.1. 100μL of sample was placed in a 10mL serum vial sealed with a butyl rubber vaccine stopper. 900μL of a 0.1M stannous chloride solution in concentrated hydrochloric acid was added and the vials were incubated at 90°C for 2 hours. Vials were allowed to cool before determination of DMS in the headspace. Calibration curves were constructed in the region of 800nM – 8mM DMSO (Li *et al.* 2007a).

2.3.2.3 Dimethylsulfone

$DMSO_2$ was quantified in solution by reduction to MT, which was measured as outlined in 2.3.2.1. A reducing reagent ("ZAV") was prepared from zinc amalgam and vanadyl sulfate, based on the mixture of Meites & Meites (1948) used for reduction of oxygen to water in air supplies. Zinc amalgam was first prepared by reacting 100g zinc powder with 100mL of 0.55M mercuric chloride solution for 2 hours at 25°C with vigorous stirring. The amalgam was washed 3 times in 5 volumes of water and then suspended in 100mL 0.1M vanadyl sulfate solution, with rapid stirring. Concentrated sulfuric acid was added immediately before use with rapid stirring, to the point at which bubbles of hydrogen appeared on the surface of the amalgam.

5mL of ZAV reagent were incubated with 1mL of the solution under test in a 30mL serum vial sealed with a butyl rubber bung at 70°C for two hours. Vials were allowed to cool to room temperature before determination of MT in the headspace. Calibration curves were constructed in the region of 5μM to 50mM $DMSO_2$.

2.3.2.4 Thiophene-3-carboxylate

A method was developed based on those of Cripps (1973) and Padden (1997) for the quantification of T2C and T2A, respectively. The molar extinction co-efficient (ε) was determined from observed A_{285} values of T3C solutions of known concentration in the range of 500μM – 50mM and was found to be 6.3mM^{-1} cm^{-1}.

From chemostat cultures, cells were removed by passage through a 0.22μm PVDF filter and A_{285} measured in a quartz cuvette. Concentrations of T3C were routinely calculated from the Beer-Lambert law.

2.3.2.5 Monomethylamine

A method for the quantification of monomethylamine (MMA) was developed based on its reaction with lactose under alkaline conditions to form a pigmented imine, as observed by Fearon (1942). This reaction has been previously exploited by Malpress & Morrison (1949) for the quantification of lactose and by Ormsby & Johnson (1950) for the quantification of MMA in urine. A novel, more sensitive, method was developed using a modification of the method of Ormsby & Johnson (1950) which allowed quantification of MMA in culture supernates in the range of 5μM – 100mM.

After the removal of cells with a 0.22μM PVDF filter, 3.5mL of the culture under test was mixed with 0.25mL 80mM lactose and 0.1mL 5M sodium hydroxide solution and was incubated at 70°C for 30 minutes and then at room temperature for a further 60 minutes. A_{545} was measured routinely and ε for the pigmented product was calculated to be 1.26mM^{-1} cm^{-1}.

2.3.2.6 Dimethylamine

Dimethylamine (DMA) was quantified using a method adapted from that of Moliner-Martínez *et al.* (2004). After removal of cells with a 0.22μM PVDF filter, 3.5mL of the culture under test were mixed with 0.25mL 760mM sodium carbonate solution and 0.25mL 92mM 1,2-napthoquinone-4-sulfonic acid solution. The reaction mixture was diluted to 10mL and A_{480} was measured routinely. DMA concentrations were calculated from a calibration curve prepared from standards in the 100nM – 100mM range.

2.3.2.7 Trimethylamine

Trimethylamine (TMA) was quantified using a method based on that of Dyer (1959). After removal of cells with a 0.22μM PVDF filter, 5mL of the culture under test was mixed with 10mL 1.3M formaldehyde solution, 10mL toluene and 3mL 2.4M sodium carbonate solution. After vigorous shaking and allowing phases to separate, 2.5mL of the toluene layer was removed and dried over sodium sulfate. The toluene solution was decanted and reacted with 2.5mL 8.6mM picric acid solution in toluene. A_{410} was measured from a glass cuvette and TMA concentrations were calculated from a calibration curve prepared from standards in the 1μM – 100mM range.

2.3.3 Quantification of inorganic sulfur compounds

Further details of methodological considerations in the quantification of inorganic sulfur compounds are given in Chapter 3.

2.3.3.1 Thiosulfate, trithionate and tetrathionate

Thiosulfate, trithionate and tetrathionate were determined using an adaptation of the cyanolytic method of Kelly *et al.* (1969). The original buffer was replaced by 100mM PIPES-HCl pH 7.4 and the assay was adapted for use on the semi-micro scale. 480μL of the solution under test and 320μL 100mM PIPES-HCl pH 7.4 were mixed in three 2mL volumetric flasks (Flasks A, B and C) and cooled in an ice-water bath to 4°C. 42μL 1M potassium cyanide solution was added to each flask and the solutions mixed thoroughly before incubating Flasks A and B at 4°C for 5 minutes, at which point 120μL 100mM cupric sulfate was added to Flask B and incubation continued for a further 15 minutes. Flask C was heated in a boiling water bath for 45 minutes. Following incubation, 240μL 1.5M ferric nitrate in 5M perchloric acid (PCA) was added to each flask and the solutions allowed to equilibrate to 20°C in a waterbath before diluting to 2mL with water. A_{460} was determined and the concentration of thiocyanate in the reaction mixture was determined spectophotometrically after measuring the absorbance of the solution in each flask (A_{460}; ε = 4.4 mM^{-1} cm^{-1}; Kelly *et al.* (1969)). Concentrations of thiosulfate, trithionate and tetrathionate were derived from the thiocyanate concentration according to Kelly *et al.* (1969), briefly, the concentration of tetrathionate was equal to that of thiocyanate in Flask A; thiosulfate concentration was calculated by subtracting twice the thiocyanate concentration of Flask A from that of Flask B; trithionate was calculated by subtracting the thiocyanate concentration in Flask B from that in Flask C.

2.3.3.2 Pentathionate, hexathionate and heptathionate

The presence of S_5-S_7 polythionates was determined chromatographically (Section 2.8).

2.3.3.3 Thiocyanate

480μL of the solution under test was mixed with 320μL 100mM PIPES-HCl pH 7.4 in a 2mL volumetric flask, 240μL 1.5M ferric nitrate in 5M PCA was added and the solution diluted to 2mL. A_{460} was determined and the concentration of thiocyanate calculated using the Beer-Lambert law using $\varepsilon = 4.4$ mM^{-1} cm^{-1} (Kelly *et al.* 1969)

2.3.3.4 Sulfate

Sulfate was quantified in solution by addition of 500μL of the sample under test to 500μL of a solution of 1.7mM barium chloride and 44mM lanthanum chloride in 0.1M hydrochloric acid (PR Norris, *personal communication*). The solutions were shaken well and allowed to react overnight at room temperature. Reactions were centrifuged at 13,000 × *g* for 15 minutes at room temperature to pellet the precipitate (comprising of barium sulfate and lanthanum phosphates). The concentration of barium remaining in the supernate was quantified by atomic absorption spectroscopy ($\lambda = 553.6$nm).

2.3.3.5 Sulfite

A method was developed based on the sulfur dioxide assay of West & Gaeke (1956), omitting the initial reaction with Hg^{2+}. 1mL of the sample under test was placed in a 25mL volumetric flask and mixed with 1mL 13.3M formaldehyde solution and 1mL 5M sodium hydroxide solution and the mixture incubated at room temperature for one hour. 3mL of 1.2mM Magenta® *O* solution (Sigma-Aldrich, ≥98% purity) in 2M hydrochloric acid was then added and the solution diluted to 25mL with MilliQ water. A_{560} was determined and the concentration of sulfite was determined from a calibration curve prepared from solutions of sulfite made in MilliQ water immediately before use in the 100nM – 100mM range.

2.3.3.6 Sulfide

Soluble sulfide was measured using an adaptation of the methods of Mylon & Benoit (2001) and Cline (1969). Mixed diamine reagent (MDR) was made from 2.0g *N*, *N*-dimethyl-*p*-phenylenediamine and 3.0g ferric chloride were dissolved in 500mL 6M hydrochloric acid. 5mL of the sample under test was mixed with 5mL MDR and allowed to react at 20°C for 15 minutes. A_{670} was measured and the concentration of sulfide in the samples was calculated from a calibration curve prepared using standard sulfide solutions in the range 10nM - 100μM. Standards were freshly prepared from washed, dried crystals of sodium sulfide nonahydrate taken from a previously unopened jar in degassed MilliQ water and were stored under argon on ice in plastic bottles to prevent loss of sulfide due to adsorption to glass.

2.3.3.7 Elemental sulfur

A method was developed loosely based on the cyanolytic method of Smith (1987). 1mL of the sample under test was extracted into 2mL benzene overnight at 37°C in a tightly sealed vial incubated statically. The benzene layer was removed and dried over sodium sulfate. 1mL of the dried benzene extract was incubated for 1 hour at 4°C with 1mL 2M potassium cyanide solution with vigorous shaking. 0.5mL of the aqueous phase was removed and reacted with an equal volume of 25mM ferric chloride in 6M hydrochloric acid before measuring A_{470}. The resultant thiocyanate concentration was calculated as for thiocyanate analyses described above. Sulfur concentration (in terms of moles of sulfur atoms, rather than S_8 molecules) was calculated from a calibration curve constructed in the 200nM – 200mM range using standard suspensions of flowers of sulfur in water.

2.3.4 Determination of formaldehyde

Formaldehyde concentration was determined colorimetrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald®). A 135mM solution of Purpald® in 1M sodium hydroxide was freshly prepared for each set of assays and a new calibration curve constructed each time from formaldehyde standards. 100μL Purpald® solution was added to 200μL of the solution under test in a 1mL volumetric flask. The solution was diluted to 1mL with MilliQ water and the colour allowed to develop for 15 minutes at room temperature. A_{550} was measured and concentration of formaldehyde calculated from calibration curves prepared from standardised solutions of formaldehyde in water in the 7nM – 70mM range (Dickinson & Jacobsen 1970).

2.3.5 Determination of fructose

Fructose was routinely assayed using the FA-20 Fructose Assay Kit (Sigma), according to the manufacturer's instructions. Samples obtained from chemostat culture were passed through 0.22μm PVDF filters to remove cells and diluted 1:10 (*v/v*) with MilliQ water before assay. Manufacturer's standards were used throughout and assays were conducted in triplicate.

2.3.6 Determination of succinate

Succinate was routinely assayed using the K-SUCC Succinic Acid Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland) according to the manufacturer's instructions. Samples obtained from chemostat cultures were passed through 0.22μm PVDF filters to remove cells and diluted 1:1 (*v/v*) with MilliQ water before assay. Manufacturer's standards were used throughout and assays were conducted in triplicate.

2.3.7 Determination of nitrite

Nitrite was routinely assayed in culture supernates using Griess Reagent (Fluka) and a method based on that of Justin (1978) for removal of thiosulfate interference. To 1mL of culture supernate, 40μL 300mM cadmium sulfate solution was added and the solution incubated on ice for 20 minutes prior to the addition of 1mL

Griess Reagent and vigorous mixing. After incubating for 30 minutes at room temperature, A_{520} was determined and concentrations of nitrite were determined from a calibration curve constructed using solutions of sodium nitrite in the range of 100nM to 100mM.

2.3.8 Determination of nitrate

5mL aliquots of culture supernate were treated for 1 hour with 5% (*w/v*) zinc powder in order to reduce nitrate to nitrite, assuming a 100% conversion. Nitrite was then assayed as described in Section 2.3.7. Calibration curves were constructed using solutions of sodium nitrate in the range 100nM to 100mM.

2.3.9 Determination of ubiquinone content

The ubiquinone (UQ) content of isolates was determined as described by Wood & Kelly (1985) using solvent modifications by Jordan *et al.* (1995). Reference ubiquinones were extracted from *M. sulfidivorans* (UQ-8), *X. tagetidis* (UQ-10) and *E. coli* (UQ-8), grown on MAMS + 15mM fructose, CBS + 20mM tetrathionate or CBS + 15mM fructose, respectively.

2.3.10 Measurement of oxygen uptake

For the majority of applications, a Clarke-type oxygen electrode was used; however, in circumstances where a solid or colloidal substrate (*i.e.* elemental sulfur) was used, it was necessary to use a manometric approach to measuring oxygen uptake by cells and extracts, since solid substrates were found to adhere to the membrane of the oxygen electrode cell.

2.3.10.1 Oxygen electrode

A Clarke-type oxygen electrode (Rank Brothers LTD, Cambridge, UK) fitted with a 7mL Perspex® cell was used according to the manufacturer's instructions. The cell was held at constant temperature during assays using a thermostatted circulating water bath (Churchill Instrument Company LTD, Perivale, UK). The electrode was connected to a chart recorder and was calibrated routinely with sodium dithionite.

2.3.10.2 Manometry

Manometer fluid (MF) with a density of 1.033g/mL, intermediate in density and composition between those of Krebs (1951) and Brodie (1910), was used throughout.

Per litre:

Anhydrous sodium bromide	44.0g
Triton® X-100	0.30g
Eosin *Y*	0.30g

The resultant fluid was thoroughly degassed *in vacuo* before use in order to prevent bubble formation in the manometer. The density (ρ) was checked periodically at 25°C by routine pycnometry against mercury.

A Warburg-pattern constant-volume manometric apparatus with pre-standardised flasks of a single side-arm design was used (B Braun Melsungen AG, Melsungen, Germany). Flasks were maintained at working temperature in a constant-temperature water bath and were routinely shaken at 120 oscillations-per-minute though 4cm. Between assays, flasks were degreased in *n*-hexane and cleaned in chromic acid. Flasks were calibrated before use by the "bicarbonate method" of Umbreit *et al.* (1964). Prior to use, flasks were coated with dimethyldichlorosilane (applied as a 0.15M solution in octamethylcyclotetrasiloxane) in order to avoid adhesion of sulfur particles or protein to the glass.

Cell suspensions or extracts were placed in the main compartment of the flasks with substrates being held in the side-arm prior to mixing. 500μL of a 10M sodium hydroxide solution was placed in the centre-well (the rim of which was greased with Amojell™ snow white petrolatum (Amoco, Chicago, IL, USA) in order to prevent the solution spilling into the reaction mixture) in order to absorb any carbon dioxide evolved during the course of the assay. All joints of the apparatus were greased with high-vacuum silicone grease and flasks were firmly attached to the manometer arms with springs to prevent changes in volume during use. A thermobarometer containing the same reagents as used in the assay with cell suspensions or extracts replaced with an equivalent volume of 13.3M formaldehyde solution was used alongside each assay and manometer readings were corrected against it at each time point.

Volumes of oxygen taken up in the assay (χ) were calculated by standard methods from the alteration in reading on the open manometer arm in millimetres (h) and the flask constant ($\mathcal{K}$) using equation [2.1]

$$\chi_{O_2}^{298K} = h\mathcal{K}_{O_2}^{298K} \qquad [2.1]$$

Flask constants ($\mathcal{K}$) for each reaction were calculated from equation [2.2], where V_g represents the volume of the gas phase in the flask and manometer; V_f is the volume of the liquid phase in the flask; α is the Bunsen co-efficient of the gas under test and P_0 is the standard pressure of the system, in mm of MF.

$$\mathcal{K}_{O_2}^{298K} = \frac{V_g \times \frac{298}{273} + V_f \alpha_{O_2}^{298K}}{P_0} \qquad [2.2]$$

2.3.11 Measurement of protein concentration

The method of Bradford (1976) was used, in the form of the pre-mixed Bio-Rad Protein Assay solution (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were diluted to water to contain ≤20μg/mL protein and dispensed in 800μL aliquots into cuvettes. 200μL of the Bio-Rad Protein Assay solution was added and the solution mixed thoroughly. A_{595} was read immediately and protein concentration calculated from calibration curves prepared from volumetrically prepared solutions of bovine serum albumin (BSA).

2.3.12 Determination of ATP

2.3.12.1 Extraction of ATP from cells

Samples (2mL cell suspension) were extracted with 0.2mL 1.45M PCA containing 10μM xylenol blue (as a convenient means of ensuring PCA neutralisation, Cohen 1922) on ice for 10 minutes then brought to pH 7.4 with 1.0mL 0.3M potassium hydroxide solution. The ATP-containing clear supernate was then decanted (Kelly & Syrett 1966).

2.3.12.2 Quantification of ATP

The FL-AA Adenosine 5'-triphosphate Bioluminescent Assay Kit (Sigma) was used with some modifications. Light emission was alternatively quantified using pre-flashed[9] 100NIF medical x-ray film (Fuji Photo Film Company LTD, Tokyo, Japan). 10-well 6mm diagnostic slides (Menzel GmbH & Co KG, Braunschweig, Germany) were placed onto x-ray film in a darkroom lit using a safelight glass No. 8U filter (Fuji). 10μL ATP assay mix, 50μL 100mM arsenate buffer (pH 7.4) containing 5mM magnesium sulfate and 2mM disodium EDTAate and 30μL of MilliQ water were placed in each well before adding 20μL supernate. After an exposure time of 5 minutes, films were developed and fixed routinely according to the manufacturers' instructions. Films were then scanned using a GS-800™ Calibrated Densitometer (Bio-Rad) and spot density was compared to those generated by ATP-standards[10] using the Quantity One® software package (Bio-Rad).

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[9] Films were pre-flashed through a SYBR® Green/Gold gel stain photographic filter (Molecular Probes, Eugene, OR, USA) using the method of Laskey (1980).

[10] Prepared from manufacturer's standards pre-treated with PCA and potassium hydroxide as per the experimental samples in order to allow for any interference from perchlorate salts (Kelly 1965).

2.3.13 Cytochrome spectra

Spectra were obtained at room temperature from CFEs using an Ultrospec 3100*pro* UV/Visible Spectrophotometer (Amersham Biosciences, Uppsala, Sweden) at 298K between 400nm and 600nm. Cytochromes were oxidised with an excess of sodium hexachloroiridite and oxidised spectra were subtracted from experimental spectra to give difference spectra. Control reduced spectra were prepared using sodium dithionite.

2.3.14 Determination of chemotaxonomic properties

Basic biochemical tests (cytochrome *c* oxidase, catalase and urease activities) and morphological characterisation (Gram and acid-fast staining; staining of inclusions, capsules, endospores, flagellae and polysaccharides) were conducted according to Hanson & Phillips (1981) and Doetsch (1981), respectively.

2.4 Enzyme assays

Spectrophotometric enzyme assays were routinely conducted in an Ultrospec 3100*pro* UV/Visible Spectrophotometer (Amersham) equipped with an eight-cuvette auto-changer. Each reaction was conducted in seven-fold replicate against a blank. Cell-free extracts (CFEs) were prepared by 3 passages through a French pressure cell (120MPa) with debris removed by centrifugation (13,000 × *g*, 30 minutes). Assays were conducted at the same temperature the organism was grown at, unless otherwise stated.

2.4.1 Enzymes of organosulfur metabolism

2.4.1.1 Dimethylsulfide monooxygenase

NADH-dependent DMS monooxygenase activity was routinely assayed spectrophotometrically at 340nm. The assay was conducted in 20mM Tris-HCl buffer at pH 7.4 containing 3mM NADH, 3μM flavin mononucleotide (FMN) and 1mM DMS. 10-30μL cell-free extract (CFE) was added to initiate the reaction. Enzyme activity was expressed in nmol NADH oxidised min^{-1} (mg protein)$^{-1}$. CFE prepared from DMSO$_2$-grown *H. sulfonivorans* S1^T was used as a positive control.

2.4.1.2 Dimethylsulfide methyltransferase

DMS methyltransferase was assessed in terms of the oxygen-independent depletion of DMS. The assay was conducted at 25°C in a water-bath. 10mL Erlenmeyer flasks fitted with PTFE-coated Suba-seal™ bungs (Sigma) were flushed with argon for 15 minutes prior to the addition of 1mL CFE, which was then flushed further with argon for 15 minutes. DMS was added in a buffer stock solution to give a final concentration of 1mM DMS in (degassed) 20mM Tris-HCl pH 7.4. Concentration of DMS in the headspace was monitored over time.

2.4.1.3 Dimethylsulfide dehydrogenase

DMS dehydrogenase activity was assayed using a modification of the method of McDevitt *et al.* (2002). 1.0mL 500mM Tris-HCl pH 8.0, 150μL 35mM PMS, 150μL 100μM DCPIP were placed in a 3mL modified Thunberg cell (Baumberger

1933) and degassed by bubbling with nitrogen for 10 minutes. 30μL 100mM DMS solution in ethanol was placed in the bulb of the side-arm and the cell assembled. The cell was evacuated for 10 minutes before sealing and the reaction initiated by pouring the contents of the side-arm into the main chamber. A_{600} was monitored. Enzyme activity was expressed in nmol DCPIP reduced min^{-1} (mg protein)$^{-1}$. CFE prepared from DMS-grown *Rhodovulum sulfidophilum* SH1 was used as a positive control.

2.4.1.4 Methanethiol oxidase

MT oxidase activity was assayed by oxygen consumption based on Smith's (1988) modifications of the method of Suylen *et al.* (1987). Oxygen uptake was measured in a Clarke-type oxygen electrode, as outlined in Section 2.3, in a 2mL final volume. 1.7mL 20mM HEPES pH 8.2 containing 5mg/mL bovine hepatic catalase and 100μL CFE were placed in the cell and 200μL of MT solutions were injected to give final MT concentrations between 3μM and 30mM. Specific activity was expressed as nmol O_2 consumed min^{-1} (mg protein)$^{-1}$. CFE prepared from DMS-grown *T. thioparus* Tk-m was used as a positive control (Gould & Kanagawa 1992).

2.4.1.5 Dimethylsulfone reductase

150μL 1.0M Tris-HCl pH 7.2, 20μL CFE and 1030μL MilliQ water was placed in a 3mL modified Thunberg cell and degassed by bubbling with nitrogen for 10 minutes. 300μL 50mM $DMSO_2$ was placed in the bulb of the side-arm of the cell and 1.5mL 2mM dithionite-reduced methyl viologen (MV) in 50mM Tris-HCl pH 7.2 was added to the main chamber and the cell assembled. The cell was evacuated for 10 minutes before sealing and the reaction initiated by pouring the contents of the side-arm into the main chamber. A_{600} was monitored and concentration of MV calculated given that $\varepsilon = 1.13 \times 10^3$ molL^{-1} cm^{-1} (Kelly & Wood 1994). Enzyme activity was expressed in nmol MV oxidised min^{-1} (mg protein)$^{-1}$. CFE prepared from DMSO$_2$-grown *H. sulfonivorans* S1 was used as a positive control.

2.4.1.6 Dimethylsulfoxide reductase

150μL 1.0M Tris-HCl pH 7.6, 20μL CFE and 1030μL MilliQ water was placed in a 3mL modified Thunberg cell and degassed by bubbling with nitrogen for 10 minutes. 300μL 50mM DMSO was placed in the bulb of the side-arm of the cell and 1.5mL 2mM dithionite-reduced MV in 50mM Tris-HCl pH 7.6 was added to the main chamber and the cell assembled. The cell was evacuated for 10 minutes before sealing and the reaction initiated by pouring the contents of the side-arm into the main chamber. A_{600} was monitored and concentration of MV calculated given that $\varepsilon = 1.13$mM^{-1} cm^{-1} (Kelly & Wood 1994). Enzyme activity was expressed in nmol MV oxidised min^{-1} (mg protein)$^{-1}$. CFE prepared from DMSO$_2$-grown *H. sulfonivorans* S1 was used as a positive control.

2.4.1.7 Methanesulfonate monooxygenase

The assay was conducted in 30mM Tris-HCl buffer at pH 7.0 containing 1mM NADH and 1mM MSA. 10-30μL cell-free extract (CFE) was added to initiate the reaction. Enzyme activity was expressed in nmol NADH oxidised min^{-1} (mg protein)$^{-1}$. CFE prepared from MSA-grown *H. sulfonivorans* S1 was used as a positive control (Thompson 1995).

2.4.1.8 Alkanesulfonate monooxygenase

Alkanesulfonate monooxygenase activity with a range of alkanesulfonates (ethanesulfonate, *n*-propanesulfonate, *sec*-propanesulfonate, *n*-butanesulfonate, *n*-pentanesulfonate, *n*-hexanesulfonate, *n*-decanesulfonate, *n*-laurylsulfonate and *n*-myristylsulfonate) were assayed using the methanesulfonate monooxygenase assay outlined in 2.4.1.7 with 1mM final concentration of the alkanesulfonate under test in place of MSA. Specific activity was expressed in nmol NADH oxidised min^{-1} (mg protein)$^{-1}$ for each alkanesulfonate assayed.

2.4.1.9 Dimethyldisulfide reductase

NADH-dependent DMDS reductase activity was assayed in CFEs based on the whole-cell method of Smith (1988). Permeablised cells were replaced with CFE and the assay conducted under argon in 10mL Erlenmeyer flasks sealed with PTFE-coated Suba-seal™ bungs. The original buffer of Smith was replaced with 20mM Tris-HCl pH 7.4. Both depletion of DMDS and formation of MT were monitored by GC of the headspace. Specific activity was expressed in nmol MT produced min^{-1} (mg protein)$^{-1}$. CFE prepared from DMDS-grown *T. thioparus* Tk-m was used as a positive control.

2.4.2 Enzymes of inorganic sulfur metabolism

2.4.2.1 Thiosulfate dehydrogenase

The assay was conducted in 30mM PIPES-HCl buffer at pH 5.0 containing 3mM potassium ferricyanide and 1mM sodium thiosulfate. 10-30μL cell-free extract (CFE) was added to initiate the reaction. A_{420} was measured and the concentration of ferricyanide determined using the Beer-Lambert law, given that ε = 1.0 mM^{-1} cm^{-1} (Kelly & Wood 1994). Enzyme activity was given in terms of nmol ferricyanide reducted min^{-1} (mg protein)$^{-1}$ (Trudinger 1965).

2.4.2.2 Thiosulfate reductase

150μL 1.0M Tris-acetate pH 8.7, 20μL CFE and 1030μL MilliQ water was placed in a 3mL modified Thunberg cell and degassed by bubbling with nitrogen for 10 minutes. 300μL 50mM sodium thiosulfate was placed in the bulb of the side-arm of the cell and 1.5mL 2mM reduced MV in 50mM Tris-acetate pH 8.7 was added to the main chamber and the cell assembled. The cell was evacuated for 10 minutes before sealing and the reaction initiated by pouring the contents of the side-arm into the main chamber. A_{600} was monitored and concentration of MV calculated given that ε = 1.13mM^{-1} cm^{-1} (Kelly & Wood 1994). Enzyme activity was expressed in nmol MV oxidised min^{-1} (mg protein)$^{-1}$.

2.4.2.3 Rhodanese

Rhodanese activity was measured using a discontinuous method. A series of test tubes were prepared, each containing, 200μL 1.0M Tris-HCl pH 7.6, 20μL 1M sodium thiosulfate solution, 2.15mL water and 30μL CFE. After incubating for a few minutes at room temperature, 50μL 1M potassium cyanide solution was added to each tube. At 10s intervals, reactions were quenched in each tube by adding 200μL 10M formaldehyde solution. Thiocyanate was determined in each tube as described elsewhere in this chapter. Activity is expressed in nmol thiocyanate formed min^{-1} (mg protein)$^{-1}$.

2.4.2.4 Sulfur oxygenase

Sulfur oxygenase was assayed in terms of oxygen consumption using a manometric approach. Various types of sulfur preparation (*cf.* 2.1.6) were use for the assay and good agreement was found between results for *lac sulfuris* and "biologically active" sulfur. Wetted sulfur produced less reproducible results and flowers of sulfur were found to be unsuitable for this assay. 1.8mL Tris-HCl pH 7.4 was placed in the main compartment of the Warburg flask along with 0.2mL CFE. 0.5mL of the sulfur preparation was placed in the side arm. The apparatus was assembled and flushed with laboratory air for 20 minutes before sealing and equilibrating at 25°C. Reactions were initiated and deviations in *h* were monitored at 30s intervals for 15 minutes. Reactions were conducted in triplicate, means of *h* taken and corrected against the mean of *h* from triplicate thermobarometers. CFE prepared from fructose/sulfide-grown cells of *T. roseopersicina* M11 was used as a positive control. Oxygen consumption was calculated using $\alpha_{O_2}^{298K}$ of 0.0285 (Umbreit *et al.* 1964). Specific activity was expressed in nmol O_2 consumed min^{-1} (mg protein)$^{-1}$.

2.4.2.5 Trithionate hydrolase

Trithionate hydrolase activity was measured using a discontinuous method. A series of test tubes were prepared, each containing 1mL 25mM potassium phosphate and 1M ammonium sulfate solution (pH 3.0), 2.15mL water and 30μL CFE. After incubating for a few minutes at room temperature, 100μL 1M sodium trithionate solution was added to each tube. At 10s intervals, reactions were quenched in each tube by adding 200μL 10M formaldehyde solution. Thiosulfate was determined in each tube as described elsewhere in this chapter. Activity is expressed in nmol thiosulfate formed min^{-1} (mg protein)$^{-1}$ (Kelly & Wood 1994).

2.4.2.6 Sulfite reductase

150μL 1.0M Tris-acetate pH 7.0, 20μL CFE, 100μL 50mM dithioerythritol and 930μL MilliQ water were placed in a 3mL modified Thunberg cell and degassed by bubbling with nitrogen for 10 minutes. 100μL 100mM sodium sulfite solution in 50mM Tris-acetate pH 7.0 containing 5mM EDTA was placed in the bulb of the side-arm of the cell and 1.5mL 2mM reduced MV in 50mM Tris-acetate pH 7.0 was added to the main chamber and the cell assembled. The cell was evacuated for 10 minutes before sealing and the reaction initiated by pouring the contents of the side-arm into the main chamber. A_{600} was monitored and concentration of MV calculated given that ε = 1.13 mM^{-1} cm^{-1} (Kelly & Wood 1994). Enzyme activity was expressed in nmol MV oxidised min^{-1} (mg protein)$^{-1}$ (Dahl *et al.* 1994).

2.4.2.7 Thiocyanate hydrolase

1mL 100mM Tris-HCl pH 7.5 buffer was placed in a 25mL serum bottle with 100μL 200mM potassium thiocyanate solution. The bottle was sealed with a butyl rubber vaccine stopper and equilibrated to 30°C before injecting with 100μL CFE. The formation of carbonyl sulfide was monitored in the headspace at 2 minute intervals for 20 minutes. Activity was expressed in nmol COS formed min^{-1} (mg $\text{protein})^{-1}$ (Kelly & Wood 1994).

2.4.2.8 Sulfite dehydrogenase

1mL 300mM Tris-HCl pH 7.2, 100μL 3mM potassium ferricyanide solution and 50μL 9mM sodium sulfite solution and 1.85mL water were placed in a 3mL cuvette and 50μL CFE added. The decrease in A_{420} was measured and the rate of ferricyanide reduction was calculated given that $\varepsilon = 1.0\ \text{mM}^{-1}\ \text{cm}^{-1}$ (Kelly & Wood 1994). Activity was expressed in terms of nmol ferricyanide reduced min^{-1} (mg $\text{protein})^{-1}$ (Kelly & Wood 1994).

2.4.2.9 Sulfide dehydrogenase

Sulfide dehydrogenase activity was measured using a discontinuous method. A series of test tubes were prepared, each contianing 1mL 500mM Tris-HCl pH 7.5, 2.15mL water and 30μL CFE. After incubating for a few minutes at room temperature, 100μL 1M sodium sulfide solution was added to each tube. At 10s intervals, reactions were quenched in each tube by adding 200μL 10M formaldehyde solution. Sulfide was determined in each tube as described elsewhere in this chapter. Activity is expressed in nmol sulfide oxidised min^{-1} (mg $protein)^{-1}$.

2.4.2.10 Sulfide oxygenase

Sulfide oxygenase was assayed in terms of oxygen consumption using a manometric approach. 1.8mL 50mM Tris-HCl pH 7.4 was placed in the main compartment of the Warburg flask along with 0.2mL CFE. 0.5mL 100mM sodium sulfide solution was placed in the side arm. The apparatus was assembled and flushed with laboratory air for 20 minutes before sealing and equilibrating at 25°C. Reactions were initiated by tipping the contents of the side-arm into the main compartment of the flask and deviations in h were monitored at 30s intervals for 15 minutes. Reactions were conducted in triplicate, means of h taken and corrected against the mean of h from triplicate thermobarometers. CFE prepared from fructose/sulfide-grown cells of *T. roseopersicina* M11 was used as a positive control. Oxygen consumption was calculated using $\alpha_{O_2}^{298K}$ of 0.0285 (Umbreit *et al.* 1964). Specific activity was expressed in nmol O_2 consumed min^{-1} (mg $protein)^{-1}$.

2.4.2.11 Adenylyl sulfate reductase

Adenylyl sulfate reductase (APS reductase) activity was routinely assayed in 3mL glass cuvettes containing 1mL 300mM Tris-HCl pH 7.4, 1mL 30mM potassium ferricyanide and 50μL of a 180mM sodium sulfite solution (freshly prepared in 100mM Tris-HCl pH 7.6 containing 10mM EDTA). 20μL CFE was added and the mixture incubated for 10 minutes at 25°C before the addition of 50μL 45mM adenosine monophosphate (AMP). A_{420} was monitored and concentration of ferricyanide calculated given that $\varepsilon = 1.00 \text{ mM}^{-1} \text{ cm}^{-1}$ (Kelly & Wood 1994). Enzyme activity was expressed in nmol ferricyanide oxidised min^{-1} (mg protein)$^{-1}$. CFE prepared from $DMSO_2$-grown *H. sulfonivorans* S1 was used as a positive control.

2.4.3 Enzymes of one-carbon metabolism

2.4.3.1 Methanol dehydrogenase

166μL 600mM Tris-HCl pH 9.0, 33μL 450mM ammonium chloride, 33μL 2.4mM DCPIP and 33μL 300mM methanol solution were placed in a 1mL cuvette with 20μL CFE and 714μL MilliQ water. The reaction was initiated by the addition of 33μL 33mM phenazine methosulfate (PMS). A_{600} was monitored and concentration of DCPIP calculated given that $\varepsilon = 21.5\text{mM}^{-1} \text{ cm}^{-1}$ (Kelly & Wood 1994). Specific activity was expressed in nmol DCPIP reduced min^{-1} (mg protein)$^{-1}$. CFE prepared from methane-grown cells of *Methylosinus trichosporium* OB3b was used as a positive control.

2.4.3.2 Formaldehyde dehydrogenase

Formaldehyde dehydrogenase activity was measured in an identical manner to methanol dehydrogenase activity, using 100mM formaldehyde solution in place of methanol solution.

2.4.3.3 Formate dehydrogenase

100μL 0.5M Tris-HCl pH 7.0, 100μL 10mM potassium ferricyanide[11], 100μL 1mM NAD+, 20μL CFE and 580μL MilliQ water were placed in a 1mL cuvette. Endogenous ferricyanide reduction was monitored at 420nm for 10 minutes at which point 100μL 100mM sodium formate was added (Moosvi *et al.* 2005). A_{420} was monitored and concentration of ferricyanide calculated given that ε = 1mM^{-1} cm^{-1}. Specific activity was expressed in nmol ferricyanide reduced min^{-1} (mg protein)$^{-1}$ (Kelly & Wood 1994). CFE prepared from methane-grown cells of *Methylosinus trichosporium* OB3b was used as a positive control.

2.4.3.4 Hydroxypyruvate reductase

100μL 500mM Tris-HCl pH 7.5, 100μL 40mM ammonium sulfate, 100μL 4mM NADH and 500μL water were placed in a 1mL cuvette and 100μL CFE added. Endogenous NADH oxidation was monitored in terms of a decrease in A_{340} before initiating the reaction by adding 100μL 2mM lithium hydroxypyruvate solution and monitoring A_{340}. NADH concentrations were derived given that ε = 3.4mM^{-1} cm^{-1} (Krema & Lidstrom 1990). Activity is expressed in terms of nmol NADH oxidised min^{-1} (mg protein)$^{-1}$ (Krema & Lidstrom 1990).

2.4.3.5 3-hexulose phosphate synthase

3-hexulose phosphate synthase activity was measured using a discontinuous method. A series of test tubes were prepared, each containing 1mL 500mM Tris-

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[11] Ferricyanide solutions were prepared immediately before use to prevent inhibition by cyanide ions (Craine & Connelly 1970).

HCl pH 7.0, 2.15mL water and 30μL CFE. After incubating for a few minutes at room temperature, 50μL 100mM formaldehyde solution and 50μL D-ribose 5-phosphate were added to each tube. At 10s intervals, reactions were quenched in each tube by adding 500μL hot ethanol. Formaldehyde was determined in each tube as described elsewhere in this chapter. Activity is expressed in nmol formaldehyde oxidised min^{-1} (mg protein)$^{-1}$.

2.4.3.6 Monomethylamine dehydrogenase

Monomethylamine dehydrogenase activity was measured in an identical manner to methanol dehydrogenase activity, using 100mM monomethylamine hydrochloride solution in place of methanol solution.

2.4.3.7 Dimethylamine dehydrogenase

Dimethylamine dehydrogenase activity was measured in an identical manner to methanol dehydrogenase activity, using 50mM dimethylamine hydrochloride solution in place of methanol solution.

2.4.3.8 Trimethylamine dehydrogenase

Trimethylamine dehydrogenase activity was measured in an identical manner to methanol dehydrogenase activity, using 30mM trimethylamine hydrochloride solution in place of methanol solution.

2.4.4 Other enzymes

2.4.4.1 Catalase

Catalase was assayed in terms of oxygen production using a manometric approach. 1.8mL Tris-HCl pH 7.4 was placed in the main compartment of the Warburg flask along with 0.2mL CFE. 0.5mL 10mM hydrogen peroxide solution was placed in the side arm. The apparatus was assembled and flushed with laboratory air for 20 minutes before sealing and equilibrating at 25°C. Reactions were initiated and deviations in *h* were monitored at 10s intervals for 5 minutes. Reactions were conducted in triplicate, means of *h* taken and corrected against the mean of *h* from triplicate thermobarometers. Oxygen production was calculated using $\alpha_{\mathrm{O_2}}^{298\mathrm{K}}$ of 0.0285 (Umbreit *et al.* 1964). Specific activity was expressed in nmol O_2 produced min^{-1} (mg protein)$^{-1}$.

2.4.4.2 Nitrilotriacetate monooxygenase

NADH-dependent NTA monooxygenase activity was routinely assayed spectrophotometrically at 340nm. The assay was conducted in 20mM Tris-HCl buffer at pH 7.4 containing 3mM NADH, 3μM flavin mononucleotide (FMN) and 1mM trisodium nitrilotriacetate. 50μL cell-free extract (CFE) was added to initiate the reaction. Enzyme activity was expressed in nmol NADH oxidised min^{-1} (mg protein)$^{-1}$.

2.4.4.3 Malate dehydrogenase

Malate dehydrogenase activity was measured in terms of the reverse reaction of the enzyme, *i.e.* the oxaloacetic acid dependent oxidation of NADH. The assay was conducted in 100mM Tris-HCl buffer at pH 7.4 containing 2mM NADH and 1mM oxaloacetic acid. NADH oxidation was measured in terms of the decrease in A_{340}. Enzyme activity was expressed in nmol NADH oxidised min^{-1} (mg protein)$^{-1}$.

2.5 Stable-isotope probing

Stable-isotope probing (SIP) experiments using [$^{13}C_2$]-DMS were conducted using the general method as follows. For concentrations of substrate, volumes of water and incubation times, see the specific experimental sections, 2.5.1 and 2.5.2.

Water samples were incubated with [$^{13}C_2$]-DMS glass serum bottles sealed with butyl rubber septa. Biomass was harvested after completed incubation periods by passage through Millipore® Sterivex™ PVDF filters with 0.22μm pore size. Filter units were stored at -80°C until required. DNA was extracted from biomass directly from within filter units using the method of Neufeld *et al.* (2008). Aliquots of DNA (1-5μg) were mixed with cesium chloride solution to give a final volume of 5.1mL with a mean density of 1.725g mL^{-1} and subjected to isopycnic ultracentrifugation at 177,000 × *g* for 40h at 20°C. Cesium chloride gradients were fractionated and DNA precipitated from [$^{13}C_n$]-DNA and [$^{12}C_n$]-DNA fractions as described by Neufeld *et al.* (2008). Partial 16S ribosomal RNA (*rrs*) genes were amplified using the polymerase chain reaction (PCR) with GC-clamped denaturing-gradient gel electrophoresis (DGGE) primers according to Neufeld *et* *al*. (2008). Amplicons were separated using DGGE on 10% (*w/v*) polyacrylamide gels containing a 30-70% (*w/v*) denaturant gradient (with 100% (*w/v*) denaturant representing 7M urea and 8.9M formamide) for 16h at 74V. Gels were stained, bands excised and DNA re-amplified, purified and sequenced according to Neufeld *et al.* (2008).

2.5.1 English Channel (UK), November 2005

Water was obtained from L4 sampling station, English Channel, UK and 750mL aliquots were placed in 1L serum bottles sealed with butyl rubber septa. MAMS medium was added to a final concentration of 0.1% (*v/v*) and samples were supplemented with [$^{13}C_2$]-DMS to a final concentration of 250μM. Control bottles were also prepared in which no substrate was added. Bottles were incubated in the dark at 19°C and the DMS concentration in the headspace monitored by GC. Once DMS had been totally consumed (5 days), bottles were opened and biomass harvested as described in Section 2.5.

2.5.2 Phytoplankton bloom transect, English Channel (UK), July 2006

The experiment was performed as described in Section 2.5.1 using water obtained from the edge of a phytoplankton bloom. Full experimental details are given in Neufeld *et al.* (2008).

2.6 Protein techniques

2.6.1 Polyacrylamide gel electrophoresis

Gels were routinely stained using 0.1% (*w/v*) Coomassie Brilliant Blue R-250 in 40% (*v/v*) methanol containing 10% (*v/v*) acetic acid and destained in 50% (*v/v*) methanol containing 5% (*v/v*) acetic acid. For some applications, the PlusOne™ Silver Staining Kit (Pharmacia) was used, using the recommended protocol for mass spectrometry applications. The PageRuler™ Plus Prestained Protein Ladder (Fermentas) was used, after calibration against standard proteins.

2.6.1.1 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely conducted using the XCell SureLock™ Cell (Invitrogen) and Novex® mini-gel cassettes routinely cast with 12.5% (*w/v*) polyacrylamide resolving gels and 4% (*w/v*) polyacrylamide stacking gels (Laemmli 1970).

2.6.1.2 NuPAGE®

10% (*w/v*) polyacrylamide 2,2-Bis(hydroxymethyl)-2,2',2"-nitrotriethanol-*N*-[Tris(hydroxymethyl)methyl]glycine (Bis-Tricine) Novex® NuPAGE® gels (Invitrogen) were used and run using the MOPS buffering system with antioxidant, according to the manufacturer's instructions.

2.6.1.3 Western Blots

Western blots were conducted from SDS-PAGE gels onto PVDF membranes using the XCell II™ Blot Module according to the manufacturer's instructions and were routinely stained with Ponceau R.

2.6.2 Proteomics

Bands were excised from PAGE gels using a sterile razor blade and were diced into 1mm × 1mm × 1mm cubes. Gel pieces were detained twice in 9.5M acetonitrile (ACN) containing 50mM ammonium bicarbonate, washed in ACN and dried at room temperature. Gel pieces were reduced with 10mM DTT and alkylated with 55mM iodoacetamide before washing with ACN, 100mM ammonium bicarbonate and a further three washes with ACN. 150ng porcine trypsin were added to each sample along with 25μL HPLC-grade water, before incubating at 37°C for 4.5 hours. Peptides were extracted from gel pieces first in 30μL 380mM ACN solution containing 265mM formic acid, followed by a second extraction with 15μL of 9M ACN solution containing 123mM formic acid. Pooled extracts were transferred to a Micromass modular CapLC and autosampler system. 6.4μL of the pooled extract was mixed with 13.6μL 26.5mM formic acid before applying onto a 0.5cm LC Packings C_{18} 5μm 100Å (Ø = 300μm) μ-precolumn cartridge. The cartridge was flushed with 26.5mM formic acid to desalt bound peptides before applying a linear gradient of 26.5mM formic acid in 18M ACN at a flow rate of 200nLmin-1 to elute peptides for further resolution on a 15cm LC Packings C_{18} 5μm 5Å (Ø = 75μm) PepMap™ analytical column, with the same gradient.

Eluted peptides were analysed on a Micromass Q-Tof Global Ultima mass spectrometer fitted with a nano-LC sprayer with an applied capillary potential of 3.5kV. The instrument was calibrated against a collisionally-induced decomposition spectrum of the doubly-charged precursor ion of human [glu^1]-fibrinopeptide B. Calibrations were accepted when the error on all subsequent acquisitions was less than 20ppm.

2.6.3 *N*-terminal sequencing

N-terminal sequences of up to 15 amino-acyl residues were obtained from Western blots of SDS-PAGE gels by Alta Biosciences (Birmingham, UK).

2.6.4 Protein purification

Protein purification steps were conducted at 4°C.

2.6.4.1 Gel filtration

Gel filtration columns were either packed manually according to the manufacturer's instructions (Sephacryl S300) or pre-packed columns were used (Superdex S200 HiLoad™). Buffers were sterilised by filtration and degassed *in vacuo* prior to use. 150mM sodium chloride was added to all gel filtration buffers to reduce protein binding to the gel matrix. The following buffers were used for purification of the enzyme:

Buffer A 20mM PIPES-HCl pH 7.4, 150mM sodium chloride, 1mM benzamidine

Buffer B 20mM PIPES-HCl pH 7.4, 150mM sodium chloride, 1mM benzamidine, 50μM ferrous ammonium sulfate, 50μM magnesium sulfate, 100mM dithiothreitol (DTT).

Buffer C 10mM PIPES-HCl pH 7.4, 5.4M glycerol.

2.6.4.2 Affinity chromatography

Small-scale binding experiments were conducted in 2mL microcentrifuge tubes with approximately 1g of a slurry of resin in buffer and 1mL of protein solution. Preparative affinity chromatography was conducted in stirred beakers at 4°C and resin was removed from the protein solution by filtration. 10% (*v/v*) glycerol was added to affinity chromatography buffers to reduce non-specific protein interactions with the matrix.

2.7 Yield estimations during methylotrophic growth

In order that theoretical Y_{ATP} could be estimated and assimilation equations for methylotrophic growth of organisms on DMS and other substrates developed, the assumptions of methylotrophic growth identified by van Dijken & Harder (1975) were applied, briefly:

I. Biomass consists of 47% (*w/w*) carbon has the formula $C_4H_8O_2N$ (Goldberg *et al.* 1976).
II. The reductant required for biosynthesis is NADH.
III. All substrate carbon is converted to biomass or carbon dioxide.
IV. The nitrogen source for growth is ammonia.

V. All biomass assimilation occurs *via* 3-phosphoglycerate (PGA).

VI. No energy is required for active transport of substrate into cells.

VII. Cells are growing at $\mu \approx \mu_{\mathrm{MAX}}$.

VIII. Assimilation of 1 PGA into biomass requires 7.25 ATP.

2.8 Thin-layer chromatography

Thin-layer chromatography (TLC) was conducted routinely using 200mm × 200mm glass-backed K6 60Å silica, 250μm TLC plates containing 254nm-sensitive fluor (Whatman). A glass chromatography tank lined with saturation pads was routinely used and the chamber equilibrated with solvent before use. All plates were developed at ambient temperature. Plates were predeveloped in solvent, dried and stored in an air-tight container before use. Samples and standards were applied (3 × 3μL) using a PTFE-tipped Hamilton syringe (Sigma) and plates were dried in a current of air between applications.

2.8.1 Development

For separation of thiosulfate and polythionates, a 1:1 (*v/v*) mixture of *n*-propanol and methanol was used (Kelly 1970); ubiquinones were developed using 20:40 (*v/v*) diethyl ether and *n*-hexane (Jordan *et al.* 1995).

2.8.2 Detection

Ubiquinones were detected by quenching of the fluor incorporated into the plates when viewed at 254nm. Thiosulfate and polythionates were detected by spraying plates with 8% (*w/v*) silver nitrate in 80% (*v/v*) acetone solution, which revealed such compounds as yellow spots, turning black upon exposure to light (modification of Kelly 1970).

2.9 General molecular biology methods

2.9.1 Genomic DNA extraction

Genomic DNA was extracted from pure cultures using the method of Feil (2004). 29.6mL bacterial cells resuspended in 10mM Tris-HCl pH 8.0 + 1mM EDTA (TE buffer) to an OD_{600} of 1.0±0.2 were incubated at room temperature for 5 minutes with 800μL 10% (*w/v*) galline egg lysozyme (Sigma) before the addition of 1.6mL 10% (*w/v*) SDS and 360μL 1% (*w/v*) fungal proteinase K (*Tritirachium album*; Melford Laboratories Ltd., Ipswich, UK) and incubating at 37°C for 1 hour. 4mL 5M sodium chloride solution was added followed by 4mL 0.27M cetyl trimethylammonium bromide (CTAB) in 0.87M sodium chloride solution and incubating at 65°C for 10 minutes. 20mL 24:1 (*v/v*) chloroform:*iso*-amyl alcohol was added and the solution mixed before centrifugation at 13,000 × *g* for 10 minutes. The aqueous phase was removed and mixed with 20mL 25:24:1 (*v/v/v*) phenol:chloroform:*iso*-amyl alcohol and the centrifugation repeated. The supernate was removed and mixed with 0.6 volumes of *iso*-propanol at -20°C and ostracine glycogen (Sigma) to a final concentration of 5ng/mL before incubating at room temperature for 30 minutes, followed by centrifugation at 13,000 × *g* for 15 minutes. The pellet was washed with 5mL 70% (*w/v*) ethanol followed by centrifugation at 13,000 × *g* for 15 minutes at which point the supernate was discarded and the pellet allowed to dry. The pellet was resuspended in 800μL TE buffer containing 0.01% (*w/v*) bovine RNAse (Sigma) and was incubated at 37°C for 30 minutes before quantification of DNA by routine agarose gel electrophoresis.

2.9.2 Quantification of DNA

DNA was quantified in solutions devoid of protein using the ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) according to the manufacturer's instructions.

2.9.3 Amplification and sequencing of the 16S ribosomal RNA gene (*rrs*)

Amplification and sequencing of the 16S ribosomal RNA gene of isolates was conducted as described by Schäfer (2007).

2.9.4 Determination of G+C content of genomic DNA

The G+C content (%mol) was determined from genomic DNA using the acetic acid method of Fredericq *et al.* (1961). Briefly, 100μL genomic DNA solution was added to 3mL 110mM acetic acid or 3mL deionised water in matched quartz cuvettes. The absorbance was measured at 230nm, 240nm, 260nm and 280nm for both treatments and the G+C content calculated (as %mol) using the set of equations given in Fredericq *et al.* (1961).

CHAPTER 3

EVALUATION OF METHODS FOR THE QUANTITATION OF SULFATE

3.1 Introduction

The quantitative analysis of inorganic sulfur compounds in both environmental samples and within laboratory cultures is of great importance to gaining a full understanding of sulfur biogeochemistry. Various methods ranging from titration through to ion chromatography using high-performance liquid chromatography (HPLC) equipment have been described in the literature for the analysis of both laboratory and environmental samples, however, very few evaluations of published techniques have been undertaken.

In previous studies, the most commonly identified end-product of dimethylsulfide (DMS) metabolism is sulfate (Chapter 1). Many colorimetric and turbidometric methods are in routine use in the microbiology laboratory for the determination of sulfate in solution; however, it should be understood that many of these methods do not work well when sulfate is present as a mixture with other inorganic sulfur compounds. The purpose of this chapter is to evaluate methods in common use and to present a more definitive method.

Throughout assessment of quantification techniques, 10μM quinine hydrochloride was used as an internal standard in all analytical standards. Concentrations of quinine were calculated using the Beer-Lambert law based on A_{348} and given that $\varepsilon = 5.7\text{mM}^{-1}\text{cm}^{-1}$ (Irvin & Irvin 1948; Allwood & Dyer 2000). All values for concentration given are corrected based upon quantification of the internal standard. Analytical standards were stored in glass serum vials under argon, in the dark at -20°C and were equilibrated to 25°C prior to use. All assays were conducted at 25°C unless otherwise stated. Anhydrous salts were stored in a desiccator over silica gel and were dried to constant weight in an oven at 70°C prior to use. Masses used to prepared standards were corrected based upon the certificates of analyses obtained from the suppliers. All assays with standards were conducted in triplicate. Sulfide standards were prepared immediately prior to use from washed and dried crystals of sodium sulfide from a newly opened jar and were handled under argon in plastic containers.

3.2 Methods in common use

A number of assays for sulfate are in routine use, ranging from precipitation-based methods to more sophisticated ion chromatographic methods which require equipment that is not available in some laboratories – particularly those that are not specialised in microbial physiology, biochemistry or environmental chemistry.

Precipitation-based methods are rapid and convenient for use in the field and in laboratories which lack ion chromatography equipment. Various methods based on the reaction of sulfate with the Ba^{2+} ion to produce a white precipitate of barium sulfate are in general use and these can be summarised as:

I) Barium precipitation and quantification of barium sulfate formed.

II) Barium precipitation and determination of residual barium in solution.

In both types of method, Ba^{2+} is typically added in the form of $BaCl_2$, though alternative procedures using $Ba(ClO_4)_2$ are also used (Haartz *et al.* 1979).

"Type I" methods are performed in two principal ways: determining $BaSO_4$ either by gravimetry or turbidometry. Schematic examples of these methods are given in Figure 3.1 (Haaijer *et al.* 2006; Norton & Peters 1993). The classical gravimetric methods are suitable only for the determination of sulfate when precent in relatively high concentrations in solution, since they are prone to loss and require use of an accurate analytical balance. Turbidometric methods are reliant upon the formation of an homogenous suspension of the $BaSO_4$ precipitate and assume that no sedimentation occurs during the determination of OD_{425}. The production of an homogenous, non-sedimenting suspension can be facilitated by the addition of 0.3% (*w/v*) gelatine to the sample prior to adding Ba^{2+} (Haaijer et al. 2006); however, reproducibility can be difficult.

In "Type II" methods, in which a standardised amount of Ba^{2+} is added to the solution under test and the residual Ba^{2+} determined, two methods are in general use, which are summarised in Figure 3.2. Colorimetric methods for determining Ba^{2+} in solution typically use disodium 3-hydroxy-4-[(E)-(2-arsonophenyl)diazenyl]naphthalene-2,7-disulfonate (Thorin) or sodium rhodizonate as indicators, measuring absorbance at 520nm in either case (Fritz & Freeland 1954). Alternatively, a more rapid and accurate determination of Ba^{2+} in solution can be achieved using atomic absorption spectroscopy (AAS) at 553.6nm (Parker 1972).

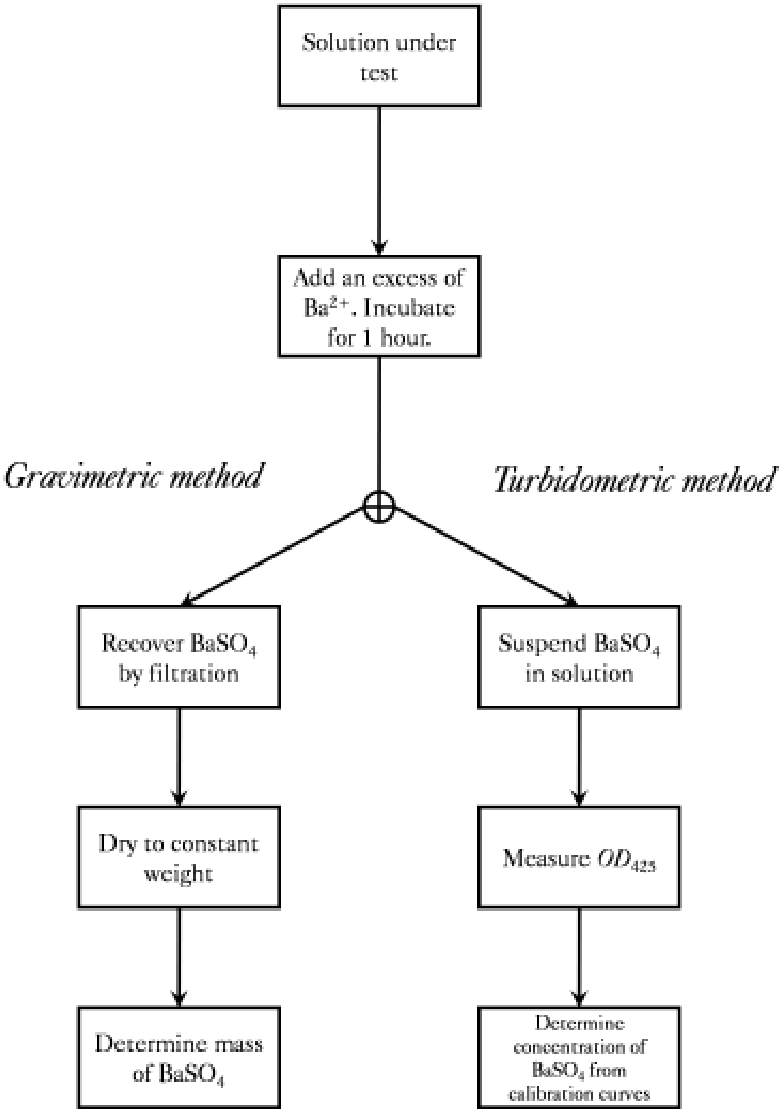

Figure 3.1 Schematic showing the gravimetric and turbidometric methods for the determination of $BaSO_4$ in the "Type I" methods of sulfate determination.

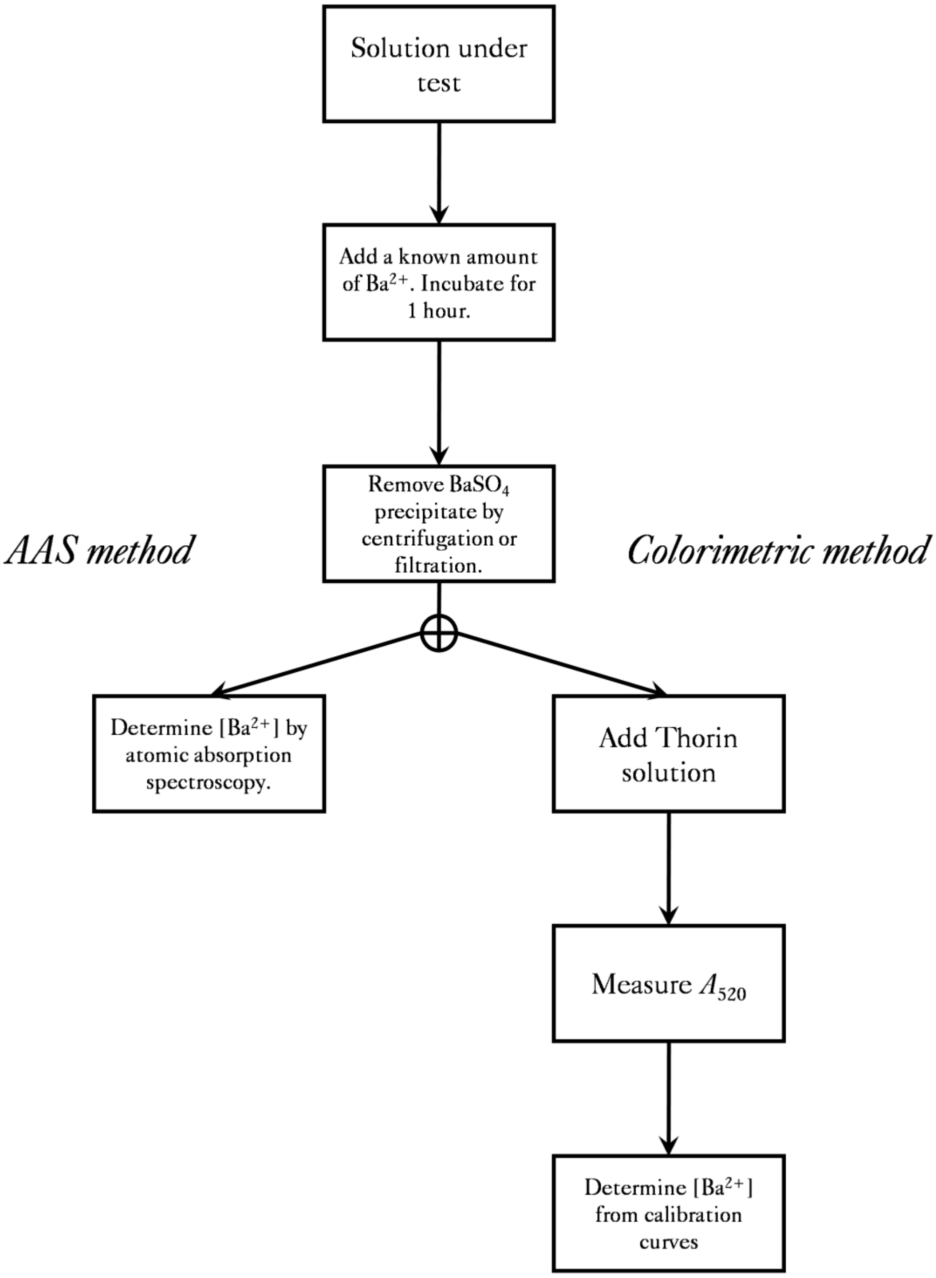

Figure 3.2 Schematic showing the atomic absorption spectroscopy and colorimetric methods for the determination of residual Ba^{2+} in the "Type II" methods of sulfate determination.

The main issue with quantification of sulfate using barium precipitation methods is the interference from other sulfur species, hydroxides, carbonates and phosphates. All of these issues can be overcome by conducting the assays at pH 1.0 and by pre-treatment with lanthanum (PR Norris, *personal communication*), since other sulfur species do not react with barium at pH 1.0, carbonates and hydroxides are not present at this pH and phosphates will precipitate in the presence of lanthanum:

$$La^{3+} + PO_4^{3-} \rightarrow LaPO_4$$

It is worth noting that at pH 1.0, any thiosulfate present in samples would break down to form elemental sulfur and sulfate. As such, samples can be pre-treated with cadmium to precipitate thiosulfate (cadmium has previously been used to remove thiosulfate from samples prior to nitrite assays by Justin (1978)).

In AAS-based methodologies, the presence of lanthanum in the sample has the added advantage of improving ionisation of barium in the flame, thus increasing the sensitivity of the method (Arslan & Tyson 1999). Additionally, lanthanum can be used as an internal standard, and can be measured by AAS at 550.1nm (Varian 1989).

In order to assess quantification techniques, a series of sulfate standards in the range 10nM – 100mM were prepared in MilliQ water from anhydrous sodium sulfate. Two forms of “interfered standard” were prepared – one containing

100μM of each sulfite, thiosulfate, tetrathionate and thiocyanate and one containing 100μM disodium hydrogen phosphate, in addition to 100mM sulfate.

For sample pre-treatment, 1 volume of the sample under test was treated with 0.1 volume 300mM cadmium chloride solution then was mixed with 1.1 volume of 0.1M lanthanum chloride in 0.1M hydrochloric acid and incubated at 4°C for 16 hours in a tightly sealed flask.

Treatment A: In the case of pre-treated samples, any precipitate was first removed by centrifugation (13,000 × *g*, 30 minutes) then filtration using a 0.4μm PVDF filter. Samples were mixed with 1 volume of 0.1M barium chloride solution and were incubated at 4°C for 16 hours. Precipitate was recovered using a dried, pre-weighed glass microfibre filter before drying and weighing.

Treatment B: Samples were mixed with 1 volume of 0.1M barium chloride and were incubated at 4°C for 16 hours. Precipitate was removed using a 0.4μm PVDF filter and the filtrate diluted to give a [Ba^{2+}] within the range of the AAS and residual barium was determined using AAS at 553.6nm.

The amounts of apparent sulfate detected from standard solutions are given, as percentages of the original concentration in Figure 3.3. It can be seen that treatment A gave poor reproducibility and was prone to error – mainly due to the difficultly in recovering and accurately weighing small masses of precipitate from the reaction mixture. Treatment B gave better reproducibility and more accurate determinations of sulfate concentration. While treatment B gave accurate determinations of sulfate in the 100nM-100mM range, treatment A was only effective at high concentrations of sulfate (≥ 10mM).

To investigate the amount of interference from phosphates and other reduced sulfur compounds on the methods of sulfate analysis, interfered standards of 100mM sulfate were assayed with treatment B, with and without pre-treatment with cadmium and lanthanum. The amounts of apparent sulfate detected in 100mM standards with and without interference are given, as percentages of the original concentration in Figure 3.4. It can be seen that without pre-treatment, the presence of phosphate gives a slightly elevated apparent sulfate concentration and that mixed reduced sulfur compounds give a large elevation with respect to the amount of sulfate present – indicating that such compounds alone may account for the "sulfate" found in various studies using this assay in the absence of lanthanum pre-treatment (*e.g.* Vila-Costa *et al.* 2006). Pre-treatment with cadmium, lanthanum and hydrochloric acid removes interference from both phosphates and reduced sulfur compounds and is therefore considered to be an absolute requirement of an accurate sulfate assay.

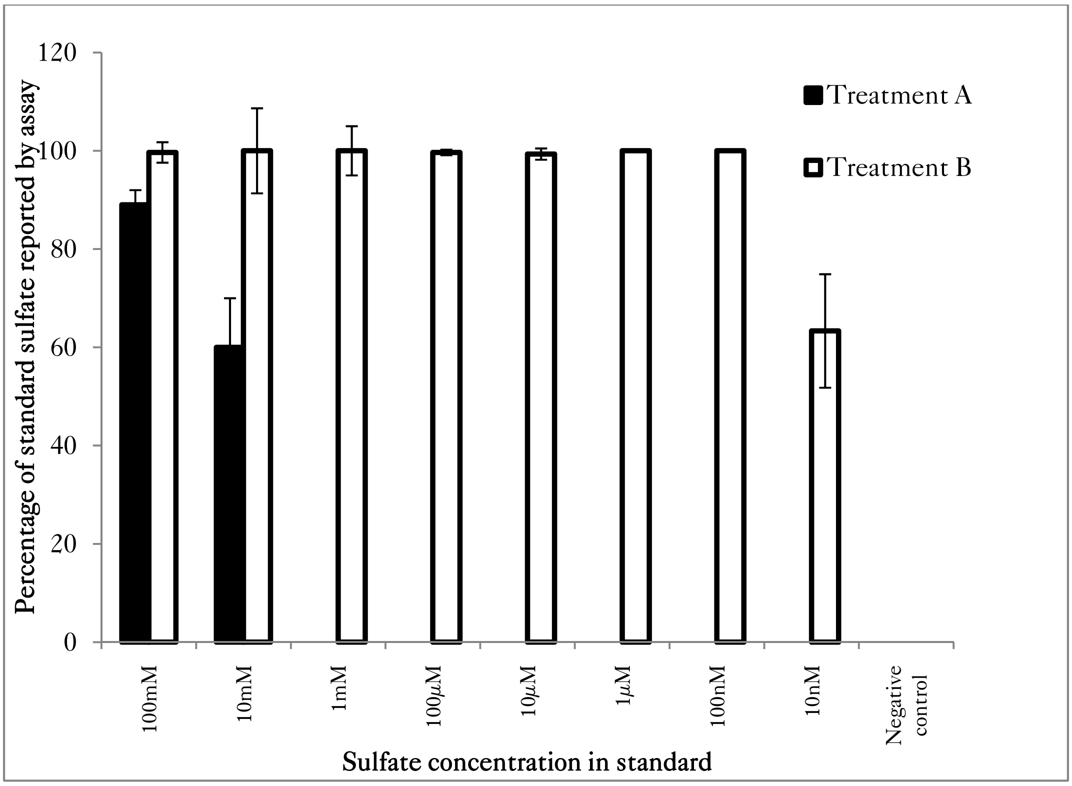

Figure 3.3 Percentages of standard sulfate apparently recovered *via* treatments A and B (see text for details) after pre-treatment with cadmium and lanthanum. Error bars indicate standard error of mean ($n = 3$).

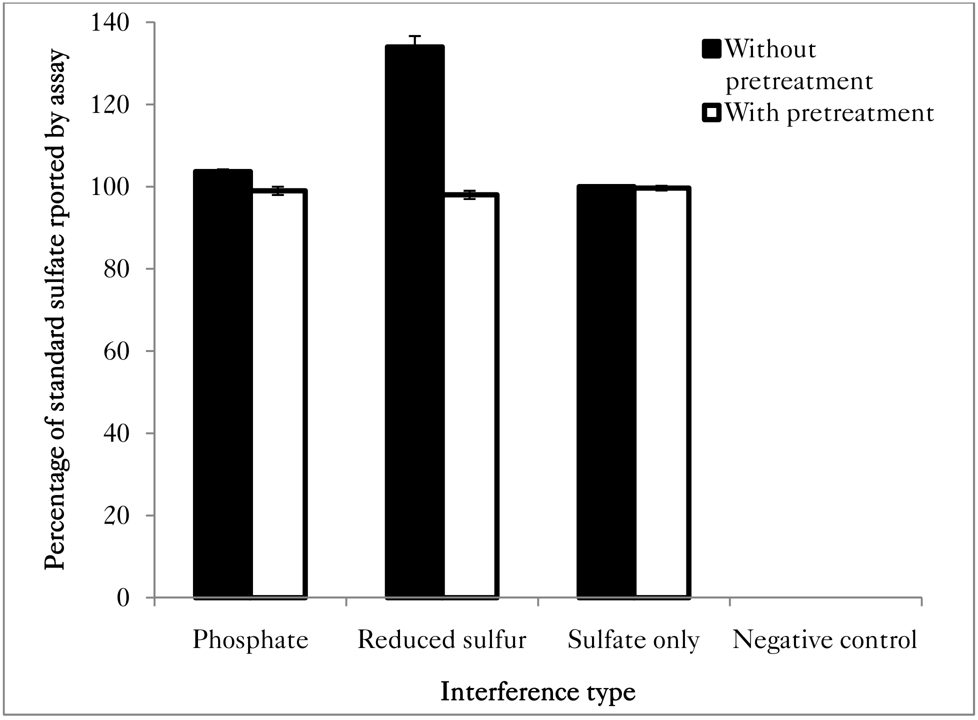

Figure 3.4 Percentages of standard sulfate apparently recovered *via* treatment B with and without pre-treatment with cadmium and lanthanum in the presence or absence of phosphate and reduced sulfur compounds (see text for details). Error bars indicate standard error of mean ($n = 3$).

3.3 Discussion

It has been shown that the quantification of sulfate using barium precipitation methods is prone to interference by phosphates and reduced sulfur compounds. It has been shown that these interferences can be simply removed by pre-treatment with cadmium (to precipitate thiosulfate) and lanthanum (to precipitate phosphates) along with conducting the assays at pH 1.0 (at which barium polythionates, barium sulfite and barium sulfide are soluble).

Precipitation-AAS based methods for indirect sulfate quantification with the sample pre-treatments given here have been shown to quantify sulfate reliably and reproducibly from 100nM to 100mM. Final volumes required for AAS vary considerably from instrument to instrument but 0.5mL was used in this study, corresponding to an absolute range of 50pmol to 50μmol sulfate required in the final solution subjected to AAS. Sulfate levels found in typical laboratory cultures of *Bacteria* grown on organosulfur compounds (in which sulfate was the end product of metabolism) would typically be in the range of 1mM to 50mM, since 1mM to 50mM substrate concentrations are typically used (Borodina 2002). Ion chromatography methods for the determination of sulfate, lower-detection limits are typically in the range of 50pmol but with a much smaller volume (in the range of 50μL) required for the assay (Bak *et al.* 1991). This would be particularly useful in, for example, discontinuous enzyme assays, in which removal of a minimal volume of the assay mixture would be necessary. Ion chromatography is a more convenient method than precipitation-AAS in terms the time taken to analyse samples and in terms of the lower sample volumes required; however, in laboratories lacking the necessary HPLC apparatus and columns required for ion chromatography, precipitation-AAS methods are a viable alternative.

The implications of the data shown in this chapter are that the traditional barium precipitation methods performed at neutral pH without sample pre-treatment can give false-positives with other sulfur species. As such, studies into the metabolism of organosulfur compounds by *Bacteria* in which these methods have been used to confirm “sulfate” as the end-product of metabolism may need to be revisited in order to determine if other compounds are present, either in addition to or instead of sulfate.

CHAPTER 4

PURIFICATION & PROPERTIES OF DIMETHYLSULFIDE MONOOXYGENASE FROM *Hyphomicrobium sulfonivorans*

4.1 Introduction

Dimethylsulfide monooxygenase activity was first described by De Bont *et al.* (1981) in *Hyphomicrobium* S, isolated from a DMSO enrichment culture inoculated with soil. No purification of the enzyme has been performed, although some basic characterisation has been achieved: the DMS monooxygenase of *T. thioparus* Tk-m has been shown to be stimulated by the presence of Ca^{2+} (Adoki 2007). Previous attempts to purify the enzyme from *Hyphomicrobium* spp. have proven difficult due to a seemingly "unstable" nature of the enzyme (H op den Camp, *personal communication*).

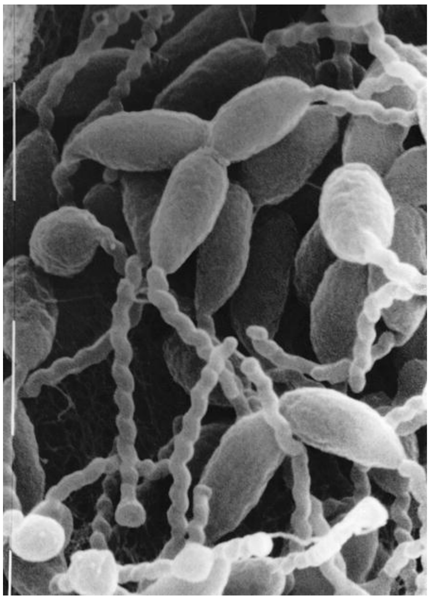

Figure 4.1. Scanning electron micrograph of *Hyphomicrobium sulfonivorans* S1^T. Scale bar = 1μm (from Borodina *et al.* 2000, Copyright © 2000 Springer-Verlag).

H. sulfonivorans S1^T (DSM 13863, ATCC BAA-113; Figure 4.1) was isolated from garden soil after enrichment on 10mM $DMSO_2$ and has been previously shown to have DMS monooxygenase activity during growth on $DMSO_2$, DMSO and DMS (Borodina 2000; Borodina *et al.* 2000; Borodina *et al.* 2002). Owing to the tolerance of *H. sulfonivorans* for high concentrations of $DMSO_2$ (up to 100mM; Borodina

2000) which allows the generation of large amounts of biomass in a short space of time, this strain was selected for an attempt at purification of the DMS monooxygenase. *H. sulfonivorans* $S1^T$ cells harvested from a total volume of 40L steady-state $DMSO_2$-limited chemostat cultures (*i.e.* 10 × 4L cultures) were pooled and disrupted at the French press (full details of growth, washing and resuspension of cells are given in Chapter 2) and the CFE cleared of debris and membranes by centrifugation at 13,000 × *g* for 30 minutes then at 150,000 × *g* for 60 minutes. A total volume of 400mL of a CFE containing 60mg/mL protein was obtained in this way. Full details of the preparation of CFEs are given in Chapter 2.

4.2 Polypeptides involves in DMS metabolism

A 54kDa polypeptide has been previously found to be induced during growth on DMS, DMSO and $DMSO_2$, which was suggested as being part of the DMS monooxygenase enzyme by Borodina (2002). Typical DMS monooxygenase activities found in previous studies of *H. sulfonivorans* were ≤10nmol NADH min^{-1} (mg protein)$^{-1}$.

4.3 Two-component model of DMS monooxygenase

4.3.1 NH_2-terminal sequencing of 54kDa peptide

Samples of the 54kDa polypeptide ("DmoA") obtained by SDS-PAGE from $DMSO_2$-grown *H. sulfonivorans* cells were blotted onto a PVDF membrane and submitted for NH_2-terminal sequencing (Alta Biosciences, Birmingham, UK). A 15-amino-acid length sequence was obtained:

Met-Lys-Lys-Arg-Ile-Val-Leu-(Asn/Asp)-Ala-Phe-Asp-Met-Thr-Cys-Val

A search of the NH_2-terminal sequence against the GenBank™ database using the BLASTp algorithm (Altschul *et al.* 1997) indicated that the sequence was related to the NH_2-termini of the large subunits of several members of the $FMNH_2$-dependent monooxygenases, including nitrilotriacetate monooxygenase (NtaA), alkanesulfonate monooxygenase (SsuD) and dibenzothiophene sulfone monooxygenase (DszA). Since $FMNH_2$-dependent monooxygenases are typically coupled to NADH-dependent FMN oxidoreductases, it was felt that a second subunit ("DmoB") would be required for optimal DMS monooxygenase activity. A re-examination of the work of Borodina (2002) showed the presence of a 19kDa polypeptide which was found to be co-expressed with DmoA during the switching of *H. sulfonivorans* from growth on methanol to DMS, DMSO or $DMSO_2$.

In order to test the hypothesis that DMS monooxygenase was $FMNH_2$-dependent, the DMS monooxygenase assay was performed on CFE prepared from cells of *H. sulfonivorans* in the presence or absence of 3μM FMN. The specific activity of DMS monooxygenase in the absence of FMN was 6.8±0.4nmol NADH oxidised min^{-1} (mg protein)$^{-1}$ ($n = 9$). When FMN was added to the assay mix, the specific activity increased to 83.4±1.9nmol NADH oxidised min^{-1} (mg protein)$^{-1}$ ($n=9$). This approximately 12-fold increase in specific activity, supports the hypothesis that DMS monooxygenase is $FMNH_2$-dependent.

4.3.2 Assay of alkanesulfonate monooxygenase and NTA monooxygenase activity in cell-free extracts

In order to determine whether the 54kDa DmoA polypeptide was part of a DMS monooxygenase or part of a related enzyme, activities of NTA monooxygenase and alkanesulfonate monooxygenase were assayed in CFE obtained from $DMSO_2$-grown *H. sulfonivorans*. Specific activities are given in Table 4.1.

Substrate	**Specific activity [nmol NADH oxidised min^{-1} (mg protein)$^{-1}$]**
Dimethylsulfide	78 (±2)
triSodium nitrilotriacetate	0 (±0)
Sodium methanesulfonate	0 (±0)
Sodium ethanesulfonate	0 (±0)
Sodium *n*-propanesulfonate	0 (±0)
Sodium *n*-butanesulfonate	0 (±0)
Sodium *n*-pentanesulfonate	0 (±0)
Sodium *n*-hexanesulfonate	6 (±3)
Sodium *n*-heptanesulfonate	0 (±0)
Sodium *n*-decanesulfonate	0 (±0)
Sodium *n*-laurylsulfonate	0 (±0)
Sodium *n*-myristylsufonate	0 (±0)

Table 4.1. Specific activities of NTA monooxygenase and alkanesulfonate monooxygenase in cell-free extracts prepared from cells of *Hyphomicrobium sulfonivorans* obtained from a $DMSO_2$-limited chemostat (D = 0.03h^{-1}, S_0 = 40mM). Figures in brackets denote standard error of mean (n = 7). "0" values refer to activities below the detection threshold.

It can be seen from Table 4.1 that no activity was observed for NTA monooxygenase or alkanesulfonate monooxygenase, excepting for a low activity with *n*-hexanesulfonate (6±3nmol NADH oxidised min^{-1} (mg protein)$^{-1}$). The *n*-hexanesulfonate monooxygenase activity was presumed to be due to the presence of impurities in the sodium *n*-hexanesulfonate (≥95% purity) as the activity was no longer present once the substrate had been recrystallised from hot water.

4.3.3 Determination of holoenzyme size by gel filtration

A Sephacryl S300 gel filtration column of 48mL bed volume was routinely calibrated in the range of 20kDa – 200kDa before equilibrating with elution buffer ("Buffer A") comprising 20mM PIPES-HCl pH 7.4, 150mM sodium chloride and 1mM benzamidine and application of 2mL of CFE. CFE was eluted with Buffer A at a flow rate of 0.5mLmin^{-1}. 0.5mL fractions were collected and assayed for DMS monooxygenase activity before analysing active fractions (found in the 70-90kDa range) by SDS-PAGE. Figure 4.2 shows SDS-PAGE analysis of the fraction with the highest activity compared to CFE, demonstrating the presence of the DmaA and DmoB polypeptides at 54kDa and 19kDa, respectively. Since the peak of enzyme activity and the densest DmoAB polypeptide bands correlated with fractions obtained in the 70-90kDa size range, it was assumed that the DMS monooxygenase was an ~73kDa protein with a structure DmoAB. Similar pairs of 54kDa/19kDa polypeptides have been previously shown to be expressed during the growth of *Hyphomicrobium* VS on DMS (Myronova, Boden & Schäfer, *unpublished data*) and of *M. podarium* on DMSO and DMSO$_2$ (Vohra 2000).

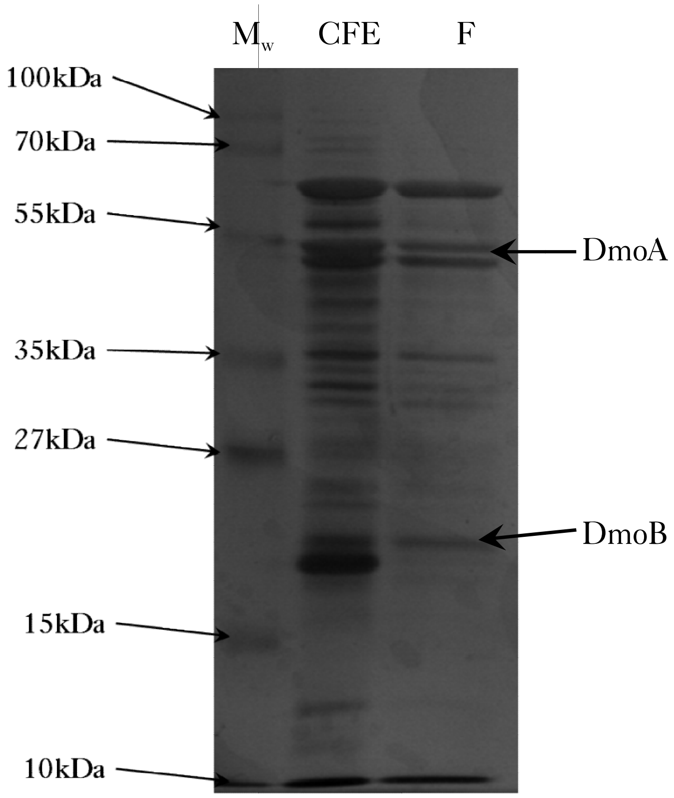

Figure 4.2. Silver-stained SDS-PAGE gel showing the gel filtration fraction with the highest DMS monooxygenase specific activity (rightmost lane – "F") compared to CFE (centre lane – "CFE") of *Hyphomicrobium sulfonivorans* grown in a $DMSO_2$-limited chemostat, with the DmoAB polypeptides indicated.

4.3.4 Determination of metal content of DMS monooxygenase

In order to assist purification, an assessment was made of metal cations associated with the enzyme activity in CFEs. Sequestration of cations from CFE samples was achieved using the EDTA method of Duewel & Woodard (2000) without modification. Divalent metal cations[12] were replaced by incubating (at a final concentration of 1mM) with sequestrated samples for 30 minutes on ice prior to assessing enzyme activity. DMS monooxygenase activities of sequestered CFE

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[12] As magnesium sulfate, calcium chloride, cupric sulfate, ferrous sulfate, cobaltous chloride, mercurous chloride, manganous sulfate, cadmium chloride or zinc chloride. Sodium sulfate or sodium chloride were used as controls and were found to have no discernable effect on the restoration of DMS monooxygenase activity.

treated with various divalent cations are given (as percentages of control activity) in Table 4.2.

Metal	None	Mg^{2+}	Ca^{2+}	Cu^{2+}	Fe^{2+}	Co^{2+}	Hg^{2+}	Mn^{2+}	Zn^{2+}	Cd^{2+}
Percentage specific activity of control	1 (±0.6)	10 (±1.2)	0 (±0.1)	1 (±0.0)	18 (±1.1)	0 (±0.0)	2 (±0.8)	2 (±0.4)	3 (±0.0)	2 (±0.2)

Table 4.2. The effect of divalent metal cations on the DMS monooxygenase activities of *H. sulfonivorans* CFE previously sequestered of metals with EDTA. DMS monooxygenase activities are given as percentages of the mean activities of an untreated control. Figures in brackets represent standard error of mean in terms of percentage activity ($n = 3$).

It can be seen from Table 4.2 that Mg^{2+} and Fe^{2+} were found to give the largest restoration of DMS monooxygenase activity in sequestered extracts. In a second experiment, Mg^{2+} and Fe^{2+} were added to sequestered extracts to a final concentration of 1mM each and incubated as previously described before assaying DMS monooxygenase activity. In this case, a 63±3% restoration of activity was observed, indicating that the monooxygenase may be dependent upon both Mg^{2+} and Fe^{2+} for optimal activity. Based on these observations, additions were made to Buffer A in order to optimise stability of the enzyme during purification – 50μM ferrous ammonium sulfate, 50μM magnesium sulfate and 100μM dithiothreitol (DTT; "Buffer B"). The Fe^{2+}/Mg^{2+} dependence of the DMS monooxygenase in *H. sulfonivorans* is in contrast to the Ca^{2+} dependence of the DMS monooxygenase from *T. thioparus* Tk-m (Adoki 2007). Whilst it would be anticipated that a different monooxygenase be present in *Thiobacillus* spp. to *Hyphomicrobium* spp., it should be noted that the studies of Adoki (2007) were conducted on whole-cell preparations of *T. thioparus* Tk-m rather than CFEs and, as such, the Ca^{2+} dependence observed may represent that of a transport protein or part of an electron transport system, rather than the DMS monooxygenase itself.

4.4 Purification of DMS monooxygenase

As can be seen from Figure 4.2, the DMS monooxygenase-containing gel filtration fractions contained various other polypeptides. By enzyme assay of the active fractions, methanol dehydrogenase, DMSO reductase and $DMSO_2$ reductase were found to be amongst the other proteins present. The gel filtration process was scaled-up using a Superdex 200 PG column (300mL bed volume) with 15mL aliquots of CFE applied each time and eluted with Buffer B at a flow rate of $2mLmin^{-1}$. Active fractions were pooled and concentrated using Amicon Ultra-15 10kDa MWCO centrifugal filter units (Millipore) before pooling concentrated fractions and assessing enzyme activity prior to a second concentration step, resulting in a solution containing essentially only proteins in the native size range of 70-80kDa. The concentrate was applied to a Sephadex G75 column (48mL bed volume) and eluted with Buffer B at $0.5mLmin^{-1}$. Active fractions were collected and assayed for DMS monooxygenase activity and by SDS-PAGE. At this stage, it was found that DMS monooxygenase was contaminated only by two peptides, in the range of 66kDa and 8kDa – these were confirmed to be the MxaF and MxaI peptides of methanol dehydrogenase by enzyme assay.

It is known that methanol dehydrogenases from *Hyphomicrobium* spp. have high affinities for hydrophobic chromatography resins such as phenyl sepharose – a property that has been used to achieve high-purity preparations of the enzyme (Arfman & Dijkhuizen 1990; Frank & Duine 1990). In order to remove MxaFI contamination from the DmoAB protein, the strong binding to phenyl sepharose was exploited. Phenyl sepharose was washed and equilibrated with 2 volumes of Buffer B before mixing 1:1 (*v/v*) with concentrated DmoAB-MxaFI solution and incubating with agitation at 4°C for 1 hour. Complexed-resin was removed from the protein solution by centrifugation (45,000 × *g*, 1 hour, 4°C). The supernate was clarified by passage through a glass fibre filter (0.45μm), concentrated using an Amicon Ultra-15 filter unit then desalted using a HighTrap™ Desalting Column (5mL; Amersham). DmoAB purified in this way was found to be essentially free of contaminating polypeptides as observed on a silver-stained NuPAGE™ gel. Table 4.3 and Figure 4.3 show the purification fold and NuPAGE™ profiles of the various purification stages.

Purification step	Protein (mg)	Activity [nmol NADH oxidised min^{-1}]	Specific activity [nmol NADH oxidised min^{-1} (mg protein)$^{-1}$]	Purification fold	% Recovery
Crude CFE	24,000	2,688,000	112	0	100
150,000 × *g*	19,040	2,341,920	123	1.1	87.1
Superdex 200 PG	9870	2,645,160	268	2.4	98.4
Amicon Ultra-15	9705	2,620,350	270	2.4	97.5
Sephadex G75	3420	2,855,700	835	7.5	106.2
Amicon Ultra-15	3340	2,772,200	830	7.4	103.0
Phenyl Sepharose	560	644,000	1150	10.3	24.0
Amicon Ultra-15	495	638,550	1290	11.5	23.8
HighTrap™ Desalting	450	565,200	1256	11.2	20.0

Table 4.3. Purification of DMS monooxygenase from *Hyphomicrobium sulfonivorans* cells obtained from a DMSO$_2$-limited chemostat.

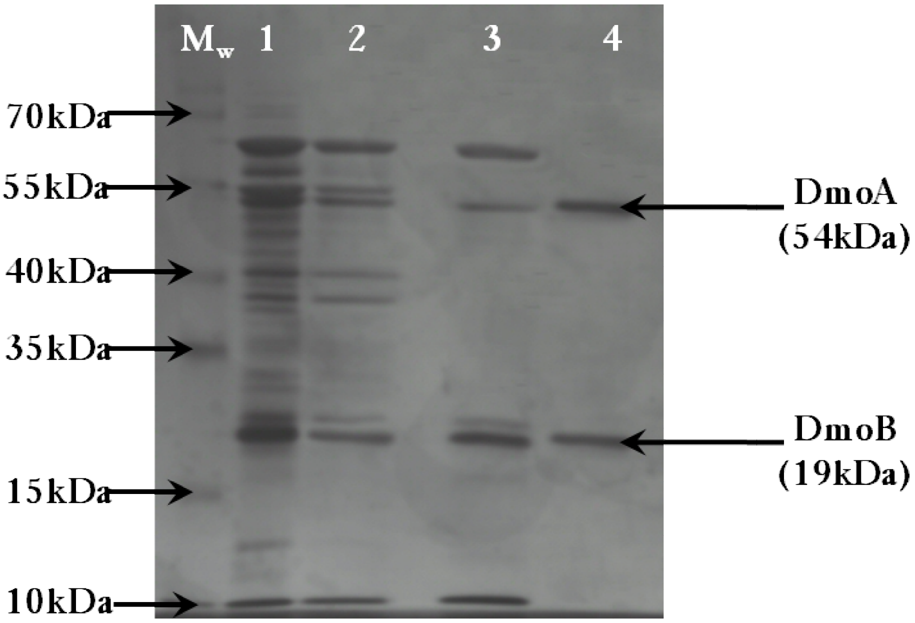

Figure 4.3. Silver-stained NuPAGE™ gel showing the purification of dimethylsulfide monooxygenase from *Hyphomicrobium sulfonivorans* grown under $DMSO_2$-limitation. 1: Soluble protein fraction; 2: Concentrated pooled active fractions from Superdex 200 PG column; 3: Pooled active fractions from Sephadex G75 column; 4: Concentrated purified protein following hydrophobic interaction chromatography with phenyl sepharose.

4.5 General properties and stability of DMS monooxygenase

Purified DMS monooxygenase was found in concentrated solution to exhibit a reddish-orange colouration, presumably due to the presence of iron in the enzyme. DMS monooxygenase was routinely stored in 10mM PIPES-HCl pH 7.4 with the addition of 50% (*v/v*) glycerol ("Buffer C") and was found to have some stability at room temperature for periods of 24 hours. Specific DMS monooxygenase activity was found to fall by approximately 80% during incubation for 24 hours in buffer C at 25°C. During characterisation, the protein was stored in aliquots in buffer C at -20°C and was used within 30 days. Activity after 30 days at -20°C was generally found to be around 90% of the original activity of a fresh preparation.

4.6 Kinetics of DMS oxidation by purified DMS monooxygenase

4.6.1 Determination of K_m and V_{MAX} for DMS

Specific activities were determined using DMS concentrations between 0.1mM and 5.0mM. V_{MAX} was determined from a Lineweaver-Burk plot (Lineweaver & Burke 1934) as 1.25μmol NADH oxidised min^{-1} (mg protein)$^{-1}$. K_m was determined from a Hanes-Woolf plot (Hanes 1932) as 16.5μM DMS.

4.6.2 Effect of alternative cofactors on DMS oxidation

In order to determine the specificity of the enzyme for NADH and $FMNH_2$, assays were conducted using combinations of NADH or NADPH with FMN, FAD, lumiflavin or lumichrome. Activities with these cofactors (given as percentages of specific activity with NADH/FMN) are shown in Table 4.4.

	FMN	**FAD**	**Lumiflavin**	**Lumichrome**
NADH	100%	64%	17%	0%
NADPH	4%	5%	2%	0%

Table 4.4. Relative specific activities of purified dimethylsulfide monooxygenase using alternative co-factor combinations. Final concentrations in the assay of nicotinamides were 1mM and flavins were 3μM.

It can be seen from Table 4.4 that NADH/FMN gave the highest enzyme activity. No activity was observed with lumichrome with either source of reducing equivalents. Activities with NADPH were much lower than those with NADH, supporting previous evidence (De Bont *et al.* 1981; Borodina *et al.* 2002) that the enzyme is NADH dependent rather than NADPH dependent. Although some activity was observed with FAD and lumiflavin in combination with NADH, it is clear from these data that NADH/FMN provide the highest activity, supporting the hypothesis that the DMS monooxygenase from *H. sulfonivorans* is a flavin-linked NADH-dependent monooxygenase.

4.7 Oxidation of thiols, sulfides and related compounds by DMS monooxygenase

4.7.1 Oxidation of thiols

No enzyme activity or substrate oxidation was observed with methanethiol, ethanethiol, *n*-propanethiol, *sec*-propanethiol, *n*-butanethiol, *sec*-butanethiol, *n*-pentanethiol, *n*-hexanethiol or thiophenol at concentrations of 0.5mM, 1mM or 2mM.

4.7.2 Oxidation of sulfides

Both symmetric (R-S-R) and asymmetric (Me-S-R) alkyl and aryl sulfides were assessed for activity at 1mM. Specific activities for alkyl sulfides are given as percentages of that with DMS in Figure 4.4. No activity was observed with diphenylsulfide or methyl phenyl sulfide.

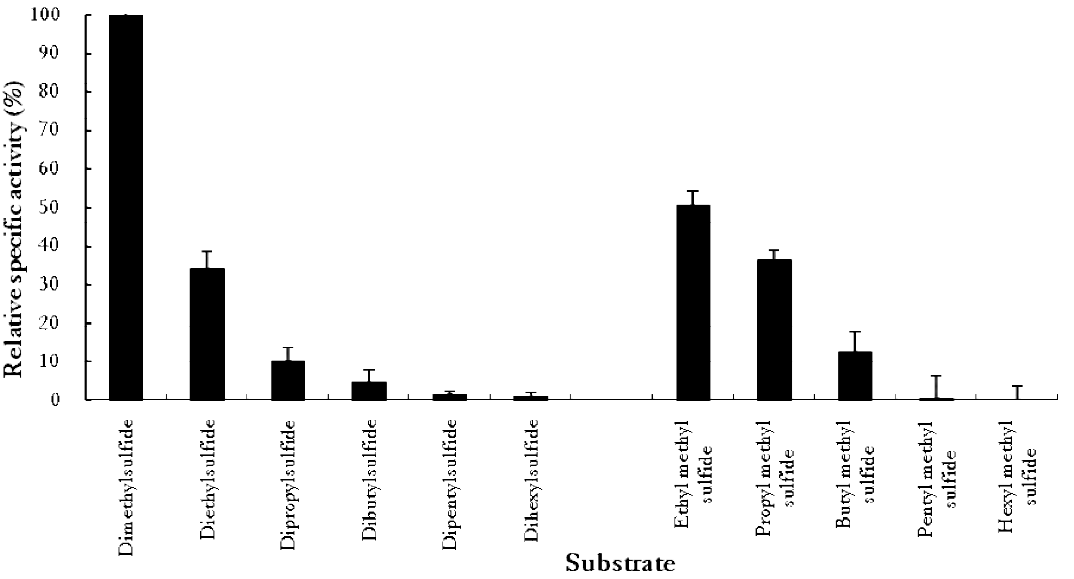

Figure 4.4. Relative specific activties of purified DMS monooxygenase with symmetric and asymmetric alkyl sulfides. Error bars indicate standard error of mean (*n* = 7).

With respect to symmetric alkyl sulfides, it can be seen from Figure 4.4 that enzyme activity is observed with all C_1-C_6 sulfides. As the alkyl chain length increases, enzyme activity is seen to decrease, with very low activity observed with dihexylsulfide. Two factors were felt to be responsible for this fall in activity – firstly the steric effects of an increased alkyl substrate chain with respect to the facility of active site binding and secondly that the dialkyl sulfides become more hydrophobic with the increase in chain length, resulting in different interactions with charged regions of the enzyme.

With respect to asymmetric alkyl sulfides, it can be seen from Figure 4.4 that, again, there is a decrease in activity with increase in chain length. The specific activity with ethyl methyl sulfide is higher than that with diethylsulfide, suggesting that oxidation of the methyl moiety of the methyl ethyl sulfide molecule can still occur, providing that the molecule is correctly orientated in the active site.

4.7.3 Oxidation of alcohols

No enzyme activity or substrate oxidation was observed with methanol, ethanol, *n*-propanol, *sec*-propanol, *n*-butanol, *sec*-butanol, *n*-pentanol, *sec*-pentanol, *n*-hexanol, *sec*-hexanol or phenol at concentrations of 0.5mM, 1mM or 2mM.

4.7.4 Oxidation of sulfonates

No enzyme activity was observed with methanesulfonate, ethanesulfonate, *n*-propanesulfonate, *sec*-propanesulfonate, *n*-pentanesulfonate, *n*-hexanesulfonate, *n*-heptanesulfonate, *n*-caprylsulfonate, *n*-laurylsulfonate or *n*-myristylsulfonate at concentrations of 0.5mM, 1mM or 2mM.

4.7.5 Oxidation of other compounds

No enzyme activity was observed with DMSO, $DMSO_2$, DMDS, phenol, naphthalene, formaldehyde or nitrilotriacetate.

4.8 Inhibition of DMS monooxygenase

4.8.1 "Classic" inhibitors of DMS metabolism

Methyl *tert*-butyl ether (MTBE) has been previously suggested as an inhibitor of DMS monooxygenase and chloroform has been proposed as an inhibitor of DMS methyltransferase (Visscher & Taylor 1993a, b). Enzyme activity was monitored using samples of purified DMS monooxygenase that had been exposed to 1mM

MTBE or chloroform for 30 minutes on ice. The specific activities observed in the presence of these compounds (70±4 and 68±4 nmol min^{-1} (mg $\text{protein})^{-1}$, respectively, *n* = 7) were of a similar magnitude to that of the control incubation (70±3 nmol min^{-1} (mg $\text{protein})^{-1}$, *n* = 7), suggesting that MTBE and chloroform did not inhibit DMS monooxygenase from *H. sulfonivorans*. It is worth noting that MTBE does inhibit the growth of *H. sulfonivorans* on DMS (E Borodina, *personal communication*), suggesting that it has an inhibitory effect on growth itself, rather than on the DMS monooxygenase enzyme. MTBE has been found to be toxic to many *Bacteria* at concentrations above 840μM (Werner *et al.* 2001).

4.8.2 Other inhibitors

DMS monooxygenase activity was assessed in samples of the purified enzyme that had been exposed to a range of inhibitors (1mM unless otherwise stated) on ice for 30 minutes. Relative specific activities are given in Figure 4.5, compared to an inhibitor-free control.

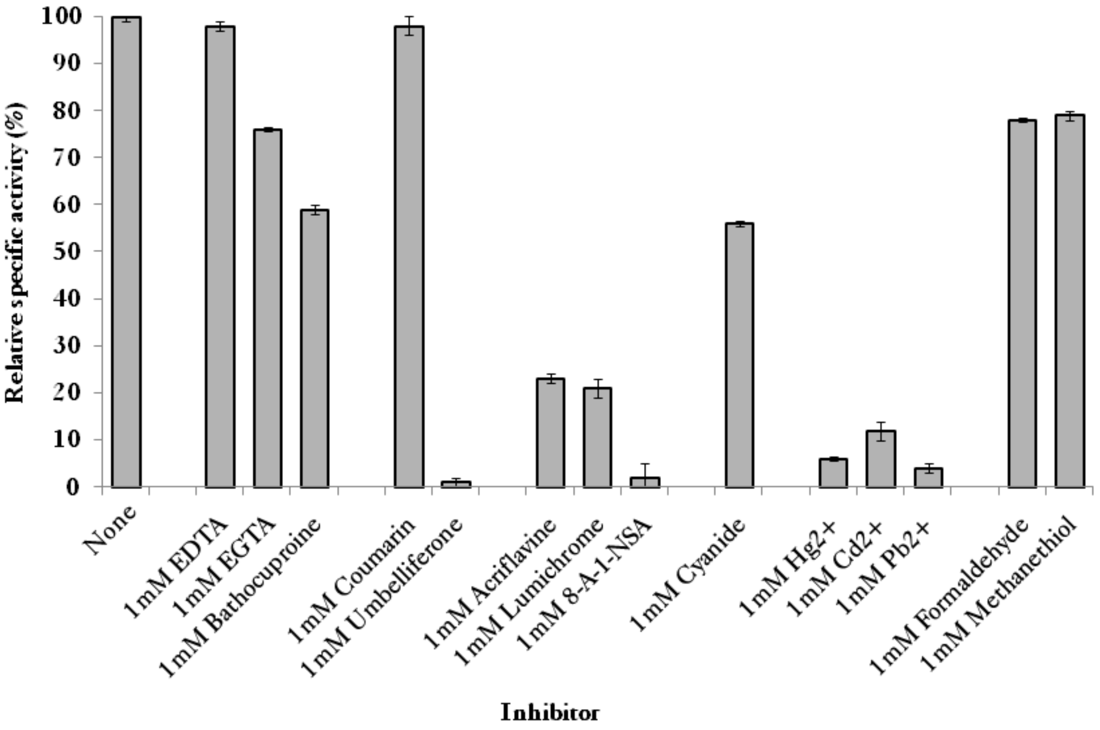

Figure 4.5. Relative specific dimethylsulfide monooxygenase activities (1mM DMS) of the purified enzyme exposed to various inhibitors for 30 minutes on ice prior to the assay. Error bars give standard error of mean ($n = 7$). Abbreviations: EDTA, disodium diaminoethanetetraacetate; EGTA, ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetracetic acid; Bathocuproine, sodium bathocuproine disulfonate; 8-A-1-NSA, sodium 8-anilino-1-naphthalenesulfonate.

It can be seen from Figure 4.5 that, of the three metal chelating inhibitors assessed (EDTA, EGTA and bathocuproine disulfonate), no inhibition occurred with EDTA. The strongest inhibition by a metal chelating agent was by 1mM bathocuproine disulfonate, which is known to chelate Cu^{2+} but not Fe^{2+} ions. Since it has been shown that Cu^{2+} does not stimulate the activity of a metal-free enzyme preparation, whereas Fe^{2+} does, it would seem unlikely that bathocuproine disulfonate was acting as a chelating agent here. It has been demonstrated previously (Ishimura & Hayaishi 1973) that bathocuproine disulfonate can inhibit oxygenase enzymes due to hydrophobic interaction with the active site – this could be the reason for the inhibition observed here.

Coumarin did not inhibit the enzyme though umbelliferone (7-hydroxycoumarin) was the strongest inhibitor found in this study. Coumarin and related compounds are known to competitively inhibit NADH-binding sites on various enzymes (BRENDA database; Schomburg *et al.* 2002), with hydroxycoumarins affording stronger inhibition in some cases. It would be assumed that umbelliferone inhibits the NADH binding site on DmoB, inhibiting the production of $FMNH_2$ required for activity of DmoA. Alternatively, umbelliferone may inhibit the enzyme in some other way, possibly by hydrophobic interaction with the active site of DmoA.

Acriflavine and lumichrome, both competitive inhibitors of FMN-binding sites (Kim & Park 2000), were found to strongly inhibit DMS monooxygenase activity, supporting the other data that suggest that the enzyme is $FMNH_2$-dependent. Sodium 8-anilino-1-naphthalenesulfonate, a hydrophobic dye used to probe active sites, has a strong inhibitory effect on the enzyme, presumably due to coating the hydrophobic regions of the active site and preventing substrate-binding.

Cyanide and heavy metals (mercury, cadmium and lead as their divalent cations) were found to inhibit the enzyme to varying degrees. 1mM cyanide inhibited the enzyme by around 45% whereas the heavy metals inhibited activity by up to 98% (Pb^{2+}). The mode of inhibition of cyanide is to bind to metal cations within enzymes, for example, in cytochrome *c* oxidase. The inhibition of DmoAB by cyanide is not particularly strong, which suggests that any metals bound to the enzyme are at least partially concealed, preventing access by the cyanide anion.

Heavy metals are known to inhibit many enzymes due to non-specific binding to negatively charged amino acids or cysteine residues, altering the enzyme conformation or blocking the active site.

The products of DMS monooxygenase (formaldehyde and methanethiol) did not inhibit the enzyme by a large degree (22% and 21% inhibition, respectively), inspite of them both being potent protein denaturants. Formaldehyde is known to form methylene cross-links within protein molecules (Hopwood 1969) and methanethiol is known to denature proteins and strongly chelate metal cations (Finkelstein & Benevenga 1986) - as such, it would be expected for both compounds to inhibit any enzyme to a high degree; however, this is not the case with DmoAB. Given the toxic nature of formaldehyde and methanethiol (and for that matter, DMS itself), the evolution of the DMS monooxygenase enzyme will have favoured a protein that is resistant to inhibition by these central metabolites in the DMS-oxidation pathway.

4.9 Molecular biology

In parallel to these studies, the genes encoding the DmoA and DmoB subunits of DMS monooxygenase were cloned from *H. sulfonivorans* and the nucleotide sequence determined (H. Schäfer, *unpublished data*). Figure 4.6 shows the structure of the gene cluster containing the *dmoA* and *dmoB* genes.

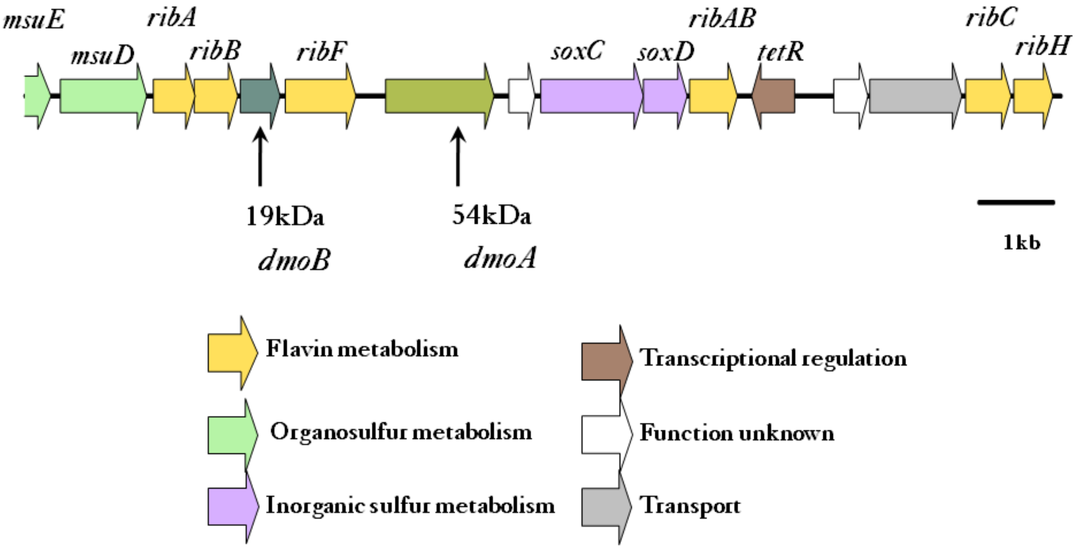

Figure 4.6. The structure of the gene cluster containing the *dmoA* and *dmoB* genes encoding the DMS monooxygenase enzyme from *Hyphomicrobium sulfonivorans*.

It can be seen from Figure 4.6 that the *dmoA* and *dmoB* genes are located in a cluster with genes of sulfur metabolism (organic and inorganic) and flavin metabolism. The MsuED polypeptides are similar to those of alkanesulfonate monooxygenases in *Pseudomonas* spp. (84% and 89%, respectively); the *ribA, ribB* and *ribH* genes encode proteins responsible for catalysing the reaction between GTP and ribulose-5-phosphate, resulting in riboflavin synthesis; the *ribC* gene encodes a flavokinase responsible for the synthesis of FMN from riboflavin; the *ribF* gene encodes an FAD synthetase (Mack *et al.* 1998). The *soxCD* genes encode proteins involved in the Kelly-Friedrich pathway of thiosulfate oxidation (Chapter 1). The presence of *msuED* genes is expected from the physiology of *H.*

sulfonivorans – it is known that the species can use alkanesulfonates as sulfur-sources during growth on methanol (Borodina 2002). The location of the *msuDE* and *dmoAB* genes in amongst genes encoding enzymes responsible for flavin metabolism is hardly surprising given that they are both $FMNH_2$-dependent enzymes. Little is known at present about gene organisation in *Hyphomicrobium* spp. and no genome sequences are available (though at the time of writing, the genome of *Hyphomicrobium denitrificans* ATCC 51888 is being sequenced by the Joint Genome Institute) so it is not known how flavin or sulfur metabolism genes are organised in other species.

In order to confirm that the DmoAB polypeptides were encoded by the *dmoAB* genes, LC-ESI-MS/MS sequencing was used, with generated peptides searched against predicted peptides generated from the gene cluster from *H. sulfonivorans*; the UniPROT™ database and the predicted proteins generated from the genome sequence of "*Methylophaga thiooxidans*". Peptide coverage maps of the amino acid sequences generated from the *dmoAB* genes are given in Figure 4.7.

DmoA:

MKKRIVLNAFDMTCVSHQSAGTWRHPSSQAARYNDLEYWTNM
AMELERGCFDCLFIADVVGVYDVYRGSAEMALRDADQVPVNDPF
GAISAMAAVTEHVGFGVTAAITFEQPYLLARRLSTLDHLTKGRVA
WNVVSSYLNSAALNIGMDQQLAHDERYEMADEYMEVMYKLWEG
SWEDDAVKRDKKSGVFTDGSKVHPINHQGKYYKVPGFHICEPSP
QRTPVIFQAGASGRGSKFAASNAEGMFILTTSVEQARQITTDIRN
QAEAAGRSRDSIKIFMLLTVITGDSDEAAEAKYQEYLSYANPEGML
ALYGGWTGIDFAKLDPDEPLQAMENDSLRTTLESLTHGENAKK
WTVRDVIRERCIGGLGPVLVGGPQKVADELERWVDEGGVDGFN
LAYAVTPGSVTDFIDYIVPELRKRGRAQDSYKPGSLRRKLIGTNDG
RVESTHPAAQYRDAYVGKESVADRTQPSPFANAKAPVAE

DmoB:

MSWTSSVSSKVMKMFSSNSSVDSDAFRSVMRQLAGCVTVITTEG
DGKLHGFTATAVCSVCAEPPSVLIAVNQTARTHPHIDKKDAFAINI
LSEDQKPLADHFATKGDDQFDTVEYALGHTGVPLLKGAAAHLE
CEVYQKIPIGTHTLFVGRVINTGQERRAPLVYYNARYGLIEQI

Figure 4.7. Peptide coverage maps of the predicted amino acid sequences of the DmoAB polypeptides showing matches (red) from peptides generated by LC-ESI-MS/MS from the purified DmoA and DmoB polypeptides after digestion with porcine trypsin.

It can be seen from Figure 4.7 that 55% coverage of the predicted DmoA amino acid sequence was achieved, along with 49% coverage of that of DmoB from tryptic peptides by LC-ESI-MS/MS analysis. These data confirm that the DmoAB polypeptides are encoded by the *dmoAB* genes.

The phylogenetic position of the DmoA polypeptide (based on the predicted amino acid sequence) amongst other $FMNH_2$-dependent monooxygenases is shown in Figure 4.8.

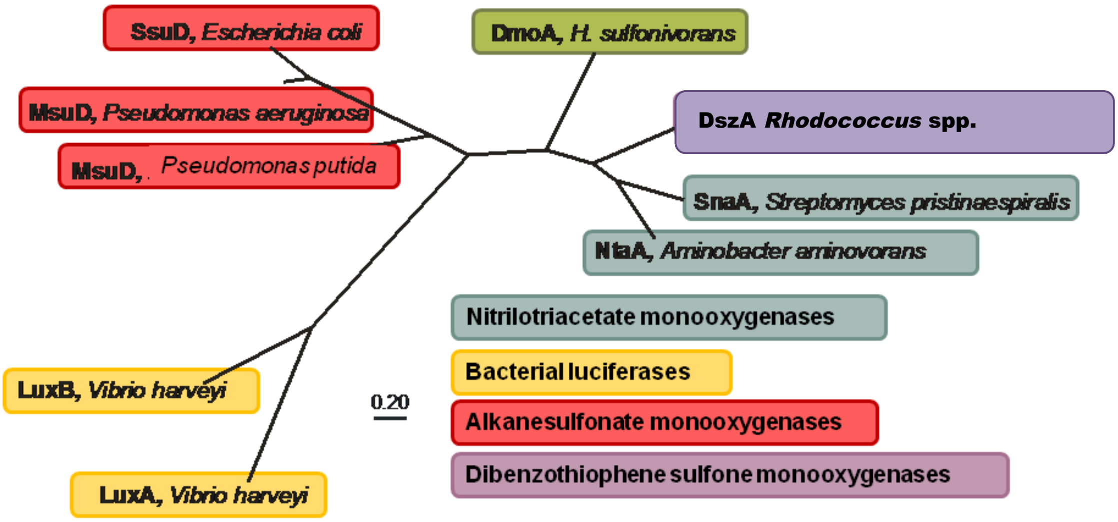

Figure 4.8. Maximum-likelyhood tree showing the phylogenetic position of the DmoA polypeptide (based on the predicted amino acid sequence) amongst the flavin-dependent monooxygenases from *Bacteria*. Scale bar indicates 0.2 amino acid substitutions per site.

It can be seen from Figure 4.8 that DmoA sits between the dibenzothiophene sulfone monooxygenase from *Rhodococcus* spp. and the alkanesulfonate monooxygenases from *Gammaproteobacteria*.

4.10 Conclusions and perspectives

Dimethylsulfide monooxygenase has been purified from *Hyphomicrobium sulfonivorans* and has been shown to comprise two subunits, a 54kDa $FMNH_2$-dependent DMS monooxygenase (DmoA) and a 19kDa NADH-dependent $FMNH_2$ oxidoreductase (DmoB). The proposed mechanism of the enzyme is shown in Figure 4.9. Although the DmoB polypeptide co-purifies with DmoA, it is not known at present if DmoB is necessary for activity or if DmoA will accept $FMNH_2$ from other oxidoreductases, such as MsuE.

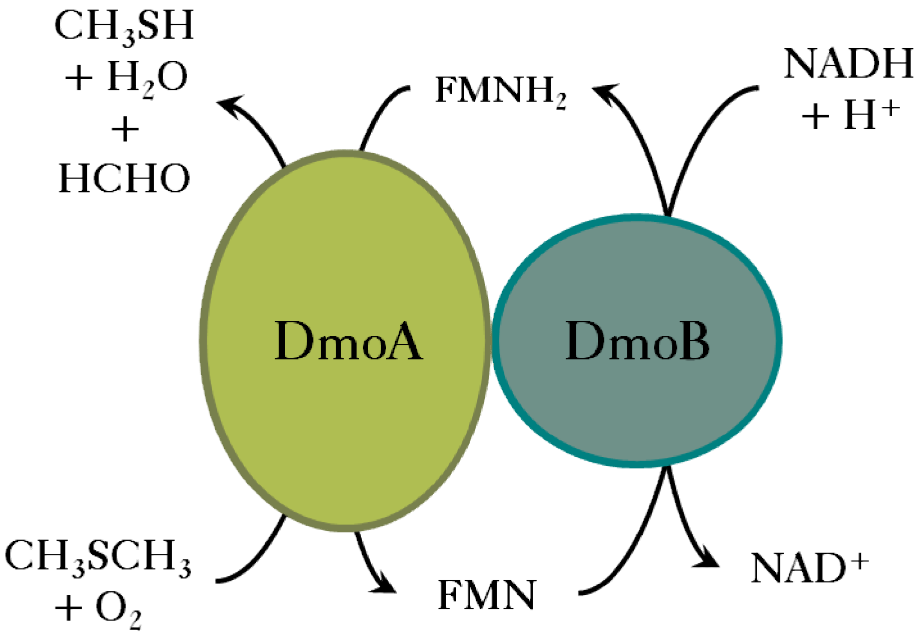

Figure 4.9. The proposed mechanism of the dimethylsulfide monooxygenase from *Hyphomicrobium sulfonivorans*.

The V_{MAX} for DMS is 1.25μmol NADH oxidised min^{-1} (mg protein)$^{-1}$ with a K_m of 16.5μM and a turnover number (K_{cat}) of 5.2s^{-1}. The DMS monooxygenase has been shown to have activity with symmetric and asymmetric alkyl sulfides but has no activity with thiols, alkanesulfonates, nitrilotriacetate, aldehydes or alcohols, indicating that this enzyme represents a new class of flavin-dependent monooxygenase in terms of function. The enzyme has been shown to be related (in terms of function and in terms of amino acid sequence) to the alkanesulfonate monooxygenases (SsuDE and MsuDE) from *Gammaproteobacteria*. It is interesting to note that dimethylsulfide monooxygenase is heterodimeric ($\alpha\beta$), whereas the alkanesulfonate monooxygenase (from *E. coli*) consists of a homotetramer of SsuD without strong association to SsuE (Eichhorn *et al.* 2002). The structure of DmoAB in terms of subunit association is similar that of bacterial luciferase (LuxAB), which is also a heterodimer. Amongst the flavin-dependent monooxygenases, three subunit arrangements are known – heterodimer (DmoAB, LuxAB), homotetramer (SsuD, EDTA monooxygenase) and homodimer (NtaA) (Eichhorn *et al.* 2002) - indicating that the subunit arrangements are not conserved throughout the family of enzymes.

The enzyme activity can be stimulated by ferrous iron and magnesium and is inhibited by heavy metals and umbelliferone. Inhibition constants have not been derived and future work on the enzyme should include studies into the mechanisms of these inhibitors in order to understand the mechanism of the enzyme. The enzyme is not inhibited by MTBE, the "classic" inhibitor of DMS monooxygenase. Further work is required to determine the location and stoichiometry of metals within the enzyme and their role in the reaction mechanism.

A more efficient purification method or a system for producing recombinant protein needs to be developed in order to facilitate further studies, particularly with respect to the crystal structure of the enzyme subunits.

Studies are underway to clone the *dmoA* gene from other organisms that possess a DMS monooxygenase (namely, *Hyphomicrobium* VS, *Methylobacterium podarium* and *Thiobacillus thioparus* Tk-m; Eyice & Schäfer, *unpublished data*) with the intention of developing suitable primers in order to be able to use the gene as a functional probe in stable-isotope probing (SIP) experiments.

CHAPTER 5

PHYSIOLOGY, BIOCHEMISTRY & BIOENERGETICS OF "*Methylophaga thiooxidans*"

5.1 Introduction

"*Methylophaga thiooxidans*" DMS010^T (DSM 22068) was isolated from an enrichment culture on 50μM DMS inoculated with a non-axenic culture of *Emiliania huxleyi* (Schäfer 2007). Preliminary studies into the metabolism of DMS by "*M. thiooxidans*" have suggested that DMS monooxygenase is not involved (Schäfer 2007) and, as such, the organism may be a suitable model organism for the DMS methyltransferase pathway. The draft assembly of the genome of "*M. thiooxidans*" has recently been completed, which gives an added advantage as a model organism. To date, "*M. thiooxidans*" is the first member of the genus *Methylophaga* to have its genome sequenced.

5.2 Physiological and biochemical characteristics of "*M. thiooxidans*"

Basic characterisation of "*M. thiooxidans*" has been carried out previously (Schäfer 2007); however, comparatively little is known about the physiology of "*M. thiooxidans*" in comparison with other *Methylophaga* spp.; basic studies were conducted into the growth physiology and biochemistry of the type strain.

5.2.1 Effect on growth by increased initial DMS concentrations

"*M. thiooxidans*" was grown in triplicate in batch culture on increasing concentrations of DMS (from 0.1mM – 10mM) and both growth and DMS consumption were followed over a 4-day period. Once cultures had reached the stationary phase, the amount of dry biomass produced (X) was determined from OD_{440}. No oxidation of DMS or increase in X were observed with DMS concentrations greater than or equal to 4.0mM, owing to the toxicity of the compound. By assessing growth at various concentrations between 3.5mM and 4.0mM, it was found that $[\mathrm{DMS}]_{\mathrm{MAX}}$ was 3.6mM. At low DMS concentrations (0.1mM, 0.5mM and 1.0mM), growth and DMS oxidation were observed, however, the most rapid initial increase in *X* was found at 2.0mM. Growth and oxidation of DMS at various concentrations are shown in Figure 5.1. The maximum DMS concentration at which growth can occur (3.6mM) is higher than the $[\mathrm{DMS}]_{\mathrm{MAX}}$ of *M. sulfidovorans* (2.4mM).

5.2.2 Range of carbon sources

"*M. thiooxidans*" has been previously shown to grow on a limited range of C_1 compounds (Schäfer 2007); however, a more extensive range of substrates had not been previously assessed. DMS010^T was capable of growth on a range of compounds (Table 5.1) including MMA, DMA, TMA, T2C, T3C, T2MA, fructose, methanol and some alkanesulfonates. Table 5.1 also contains information concerning the range of carbon sources used by other *Methylophaga* spp. based on data obtained from Janvier *et al.* (1985), De Zwart *et al.* (1996), Doronina *et al.* (2003a, b), Doronina *et al.* (2005), Kim HG *et al.* (2007) and from growth experiments performed on other substrates as part of this study.

It can be seen from Table 5.1 that all *Methylophaga* spp., including "*M. thiooxidans*", grow on a restricted range of C_1-compounds and on fructose, which is a common property of "facultative methylotrophs", regardless of the class of *Proteobacteria* to which they are affiliated. An interesting property of "*M. thiooxidans*" which is not shared with other *Methylophaga* spp. used in this study is growth on substituted thiophenes. Substituted thiophenes are compounds of pharmaceutical and environmental significance found in a variety of environments; however, their role in nature and in the sulfur cycle is poorly understood. As far as can be determined from the literature, thiophenes have not been quantified in marine environments. It is known that substituted thiophenes are produced by various plants, for example, thiophene-2-carboxylate (T2C) from *Tagetes patula* L. (French marigold) and *Tagetes erecta* L. (Mexican marigold; Jacobs *et al.* 1994) and echinothiophene from *Echinops grijissii* L. (Globe thistle; Koike *et al.* 1999). Various bacteria have been isolated from the roots of thiophene-producing plants (*e.g. Xanthobacter tagetidis* from *Tagetes* spp.; Padden *et al.* 1997) but their metabolism remains poorly understood. Some *Methylophaga* spp. have been shown to have a metabolic association with *Ulva lactuca* L. (Sea lettuce) and *Cystoseira trinodes* L. (Li TD *et al.* 2007) in which the *Methylophaga* spp. secrete indole-3-acetic acid to stimulate algal growth, resulting in a greater formation of DMS (from DMSP) and methanol (from lignin). It is possible that marine algae with which *Methylophaga* spp. are associated could secrete substituted thiophenes, as do many terrestrial plants, providing an explanation as why *Methylophaga* spp. can grow on these compounds.

The growth of "*M. thiooxidans*" (along with some other *Methylophaga* spp.) on long-chain alkanesulfonates (C_{10}, C_{12} and C_{14}) but not shorter ones (C_1, C_4 and C_5) is similar to the pattern of sulfonate utilisation previously observed in *Rhodococcus* sp. CB1 (Erdlenbruch *et al.* 2001). Since long-chain alkanesulfonates are not found in nature, the ability to grow on them may stem from mechanisms in place to oxidise complex humic sulfonates found in soils and aquatic systems (Kertesz 1999).

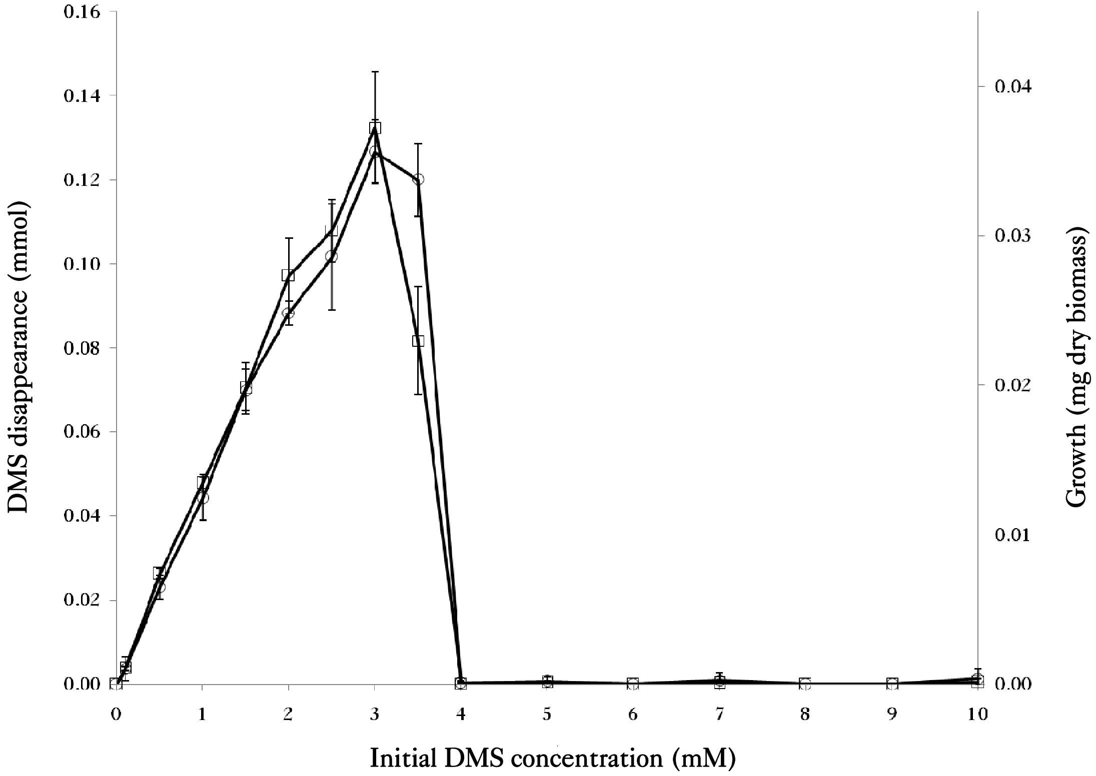

Figure 5.1 Disappearance of and growth on DMS at increasing initial concentration by "*Methylophaga thiooxidans*" after 4 days. Circles, DMS diseappeared (mmol); Squares, growth (mg dry biomass). Error bars indicate standard error of mean (*n* = 3).

Substrate (Concentration)	Growth after 14 days incubation				
	"M. thiooxidans"	*M. alcalica*	*M. aminisulfidivorans*	*M. sulfidovorans*	*M. thalassica*
DMS(2mM)	+	-	+	+	-
DMSO (20mM)	-	-	+	-	-
$DMSO_2$ (20mM)	-	-	-	-	-
CS_2 (2mM)	-	-	-	-	-
DMDS(2mM)	-	-	-	-	-
DMSP (2mM)	-	-	-	-	-
MMA (20mM)	+	+	+	+	+
DMA (10mM)	+	-	+	+	-
TMA (10mM)	+	-	+	-	-
Thiophene (2mM)	-	-	-	-	-
T2A (2mM)	-	-	-	-	-
T3A (2mM)	-	-	-	-	-
T2C (2mM)	+	-	-	-	-
T3C (2mM)	+	-	-	-	-
T2MA (2mM)	+	-	-	-	-
DBT (2mM)	-	-	-	-	-
MSA (10mM)	-	-	-	-	-
n-Propanesulfonate (10mM)	-	-	-	-	-
sec-Propanesulfonate (10mM)	-	-	-	-	-
n-Pentanesulfonate (10mM)	-	-	-	-	-
n-Caprylsulfonate (10mM)	+	-	+	+	-
n-Laurylsulfonate (10mM)	+	-	+	+	-
n-Myristylsulfonate (10mM)	+	-	+	+	-
Benzenesulfonate (1mM)	-	-	-	-	-
Acrylate (10mM)	-	*N.D.*	-	-	*N.D.*
Pyruvate (5mM)	-	*N.D.*	-	-	*N.D.*
Formate (10mM)	-	*N.D.*	-	-	*N.D.*
Malate (5mM)	-	*N.D.*	-	-	*N.D.*
Benzoate (5mM)	-	*N.D.*	-	-	*N.D.*
Tartrate (5mM)	-	*N.D.*	-	-	*N.D.*
Succinate (5mM)	-	*N.D.*	-	-	*N.D.*
Propanoate (5mM)	-	*N.D.*	-	-	*N.D.*
Glutamate (5mM)	-	*N.D.*	-	-	*N.D.*
Acetate (5mM)	-	-	-	-	-
Citrate (5mM)	-	-	-	-	-
Methanol (50mM)	+	+	+	+	+
Ethanol (25mM)	-	-	-	-	-

Substrate (Concentration)	Growth after 14 days incubation				
	“M. thiooxidans”	*M. alcalica*	*M. aminisulfidivorans*	*M. sulfidovorans*	*M. thalassica*
n-Propanol (15mM)	-	-	-	-	-
iso-Propanol (15mM)	-	-	-	-	-
n-Butanol (5mM)	-	-	-	-	-
n-Pentanol (5mM)	-	-	-	-	-
n-Hexanol (5mM)	-	-	-	-	-
n-Heptanol (5mM)	-	-	-	-	-
Urea (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Sorbitol (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Alanine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Glycine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Serine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Phenylalanine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Taurine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Cysteine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Arginine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Methionine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Glutamine (5mM)	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Xylose (5mM)	-	-	-	-	-
Arabinose (5mM)	-	-	-	-	-
Glucose (5mM)	-	-	-	-	-
Sorbose (5mM)	-	-	-	-	-
Fructose (5mM)	+	-	+	+	+
Galactose (5mM)	-	-	-	-	-
Sucrose (5mM)	-	-	-	-	-
Maltose (5mM)	-	-	-	-	-
Lactose (5mM)	-	-	-	-	-
Betaine (5mM)	-	-	-	-	-
Choline (5mM)	-	-	-	-	-
Methanethiol (2mM)	+/-	-	+/-	+/-	-
Ethanethiol (2mM)	-	-	-	-	-
n-Propanethiol (2mM)	-	-	-	-	-
sec-Propanethiol (2mM)	-	-	-	-	-
Methane (2mM)	-	-	-	-	-
Formaldehyde (0.5mM)	-	-	-	-	-
Carbon monoxide (0.5mM)	-	-	-	-	-

Table 5.1. Substrate profiles of *Methylophaga* spp. isolated to date (+ growth observed; - no growth observed; +/- weak growth observed, *N.D.* not determined).

5.2.3 Range of nitrogen sources

"*M. thiooxidans*" was grown on 20mM methanol in batch culture using N-MAMS supplemented with various nitrogen sources to 2.5mM. Growth was monitored in terms of OD_{440} after 7 days. The presence/absence of growth on each nitrogen source is detailed in Table 5.2. It can be seen that "*M. thiooxidans*" is capable of growth on ammonium, nitrate, urea or methylated amines as sole nitrogen sources. No growth was observed with other inorganic nitrogen sources. These data are in agreement with the properties of other *Methylophaga* spp. (Kim HG *et al.* 2007).

Nitrogen source	Growth
Ammonium	+
Nitrate	+
Nitrite	-
Nitrogen[13]	-
Thiocyanate	-
Cyanate	-
Urea	+
MMA	+
DMA	+
TMA	+

Table 5.2 Growth of "*Methylophaga thiooxidans*" on methanol with alternative nitrogen sources (+ growth observed; - no growth observed).

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[13] *i.e.* no nitrogen source added to the medium and nitrogen in the air in the headspace as the only nitrogen source.

Species	Strain	G+C (mol%)	Reference
M. alcalica	M39	48.3	Doronina *et al.* 2003a
M. aminisulfidivorans	MP	44.9	Kim HG *et al.* 2007
M. natronica	Bur2T	45.0	Doronina *et al.* 2003b
M. thalassica	ATCC 33146	44.0	Janvier *et al.* 1985
M. sulfidovorans	RB-1	42.0	De Zwart *et al.* 1996
M. marina	ATCC 35842	43.0	Janvier *et al.* 1985
M. murata	Kr3	44.6	Doronina *et al.* 2005
"*M. thiooxidans*"	DMS010	45.9	This study

Table 5.3. G+C contents of *Methylophaga* spp. isolated to date.

5.2.4 Determination of ubiquinones

The dominant ubiquinone in DMS- and methanol- grown cells of "*M. thiooxidans*" was found to be UQ-8, which is consistent with all other members of the genus *Methylophaga* (Kim HG *et al.* 2007).

5.2.5 Determination of G+C content

The G+C content was found to be 45.9mol% and is shown in Table 5.3 alongside the G+C contents of other members of the genus *Methylophaga*.

5.2.6 Phylogenetic position of "*Methylophaga thiooxidans*"

The 16S rRNA (*rrs*) gene sequence (DQ660915) of "*M. thiooxidans*" was aligned against those of other *Methylophaga* spp. using the CLUSTAL program. The phylogenetic relationships were determined using the neighbour-joining method with the Molecular Evolutionary Genetics Analysis (MEGA) software package with bootstrap analysis of 100 trees (Tamura *et al.* 2007).

A neighbour-joining tree showing the position of "*M. thiooxidans*" amongst other members of the genus *Methylophaga* based on 16S rRNA gene (*rrs*) is shown in Figure 5.2.

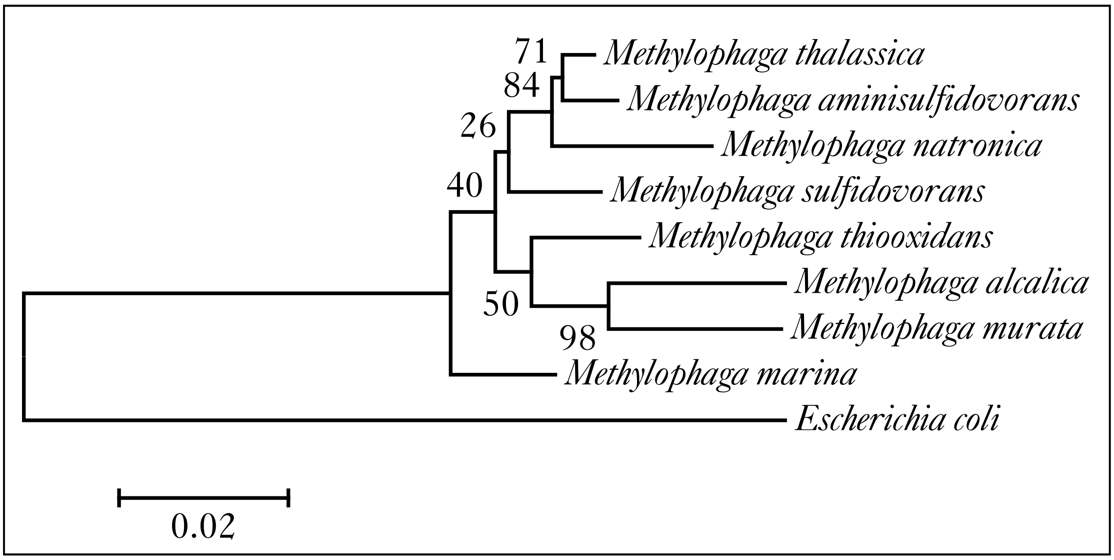

Figure 5.2. Position of "*Methylophaga thiooxidans*" within the genus *Methylophaga*. GenBank™ accession numbers of 16S rRNA gene sequences of type strains of each species: *M. thalassica* X87339; *M. aminisulfidivorans* DQ463161; *M. natronica* AY128533; *M. sulfidovorans* X95461; "*M. thiooxidans*" DQ660915; *M. alcalica* AF384373; *M. murata* AY694421; *M. marina* X87338; *E. coli* AY513502. Numbers at branch points are bootstrap values from 100 replicates. Bar: 2 nucleotide substitutions per 100.

5.3 Growth in chemostat culture

"*M. thiooxidans*" was grown in substrate-limited chemostats on both methanol and DMS and its kinetic parameters determined and compared with those of the closely related DMS-oxidising isolate *M. sulfidovorans*.

5.3.1 Under methanol-limitation

Steady-state cultures were established with 30mM methanol as the growth-limiting substrate at dilution rates (D) between 0.007 and 0.13h^{-1}, between which the steady-state growth yield (Y) increased from 8.40 to 16.02g dry biomass per mole of methanol. The maximum growth yield (Y_{max}) was estimated from a plot of

$1/Y$ *versus* $1/D$ and found to be 16.5g dry biomass per mole of methanol. The maximum growth rate (μ_{max}) was found to be $0.14h^{-1}$ by washout kinetics. The maintenance energy coefficient with respect to carbon source (m_S) was determined from a plot of specific rate of substrate uptake (q) *versus* D and was found to be 13mmol methanol $g^{-1} \cdot h$. The specific maintenance rate (a) was determined as $0.22h^{-1}$ and the growth efficiency (E_G) found to be 0.4. By comparison, the related DMS-oxidising species *M. sulfidovorans* has a higher μ_{max} on methanol of $0.30h^{-1}$ but with a lower Y_{max} of just 10.0g dry biomass per mole of methanol (De Zwart *et al.* 1996), which relates to a a of $0.03h^{-1}$ and E_G of 0.9, indicating that it is more than twice as efficient during growth on methanol as a sole carbon and energy source than "*M. thiooxidans*". It can be seen from the vastly higher value of a in "*M. thiooxidans*" that this species has a rate of turnover of biomass of over 7 times that of *M. sulfidovorans*.

Consideration of the theoretical Y_{max}, based on the models for methylotrophic growth of Anthony (1982) can be made, using the assumption that all *Methylophaga* spp. act as KDPG-variant RuMP-cycle methylotrophs during growth on methanol as the sole source of carbon and energy. The theoretical Y_{max} for "*M. thiooxidans*" or *M. sulfidovorans* growing under methanol-limitation is 18.9g dry biomass per mole of methanol. The actual Y_{max} for *M. sulfidovorans* is considerably lower (almost half) that of the theoretical Y_{max} whereas that of "*M. thiooxidans*" falls just 2.4g below that of the theoretical Y_{max}. *M. sulfidovorans* is a more efficient organism that "*M. thiooxidans*" during methanol-limited growth; however, it has an actual Y_{max} much lower than that of the theoretical Y_{max}. Aside from the obvious reasons for discrepancy between theoretical and experimental Y_{max} (*e.g.* motility), it should be noted that the model of Anthony notes that the actual Y_{max} for methanol utilisers is often much lower than the calculated values – presumably due to poor coupling of NADH dehydrogenase to ATP synthesis. If a low P/O ratio for NADH oxidation is considered in the model (*e.g.* 0.5), a lower theoretical Y_{max} of 12.3g dry biomass per mole of methanol is calculated, which is closer to the experimentally derived Y_{max} of *M. sulfidovorans*. The Y_{max} of "*M. thiooxidans*" is much higher than that of both *M. sulfidovorans* and the theoretical Y_{max} calculated using a low P/O ratio to account for poor coupling of NADH dehydrogenase to ATP synthesis. This could indicate that the coupling of NADH dehydrogenase to ATP synthesis is much more efficient in "*M. thiooxidans*", leading to a Y_{max} closer to the theoretical Y_{max} as calculated with the higher P/O ratio. Alternatively, a second methanol dehydrogenase enzyme may be present in "*M. thiooxidans*" which could allow more methanol to be assimilated to biomass. It is worth noting that the XoxF peptide, which has been implicated as an alternative methanol dehydrogenase to the classical Mxa-type dehydrogenase has been found in methanol-grown "*M. thiooxidans*" cells (Schäfer 2007) and an "unusual" second methanol dehydrogenase with different physical and kinetic properties to the Mxa-type dehydrogenase has been previously found in *M. marina* (Chan & Anthony 1992) – which could correspond with the XoxF peptide expressed in "*M. thiooxidans*". It is not currently known whether the Xox-type dehydrogenase is found in *M. sulfidovorans*.

5.3.2 Under DMS-limitation

Steady-state cultures were established with 15mM DMS as the growth-limiting substrate at $D = 0.007 - 0.07\text{h}^{-1}$, between which Y increased from 1.60 to 10.79g dry biomass per mole of DMS, equivalent to 0.80 to 5.40g per mole of DMS carbon. Y_{max}, estimated from a plot of $1/Y$ *versus* $1/D$ was found to be 14.4g dry biomass per mole of DMS (7.2g per mole of DMS carbon). From washout kinetics, μ_{max} was found to be 0.08h^{-1}. From a plot of q *versus* D, m_{S} was found to be 8mmol DMS $\text{g}^{-1}\cdot\text{h}$. By comparison, *M. sulfidovorans* has a μ_{max} of 0.04h^{-1} on DMS and a Y_{max} of 9.2g dry biomass per mole of DMS (4.6g per mole of DMS carbon; De Zwart *et al.* 1996); a was determined as 0.12h^{-1} and E_{G} found to be 0.4 – identical to the E_{G} during growth on methanol. The related DMS-oxidising species *M. sulfidovorans* has a a of 0.18h^{-1} and E_{G} of 0.3, indicating that "*M. thiooxidans*" is slightly more efficient than *M. sulfidovorans* during growth on DMS. Comparable values of a in both organisms indicate that their rates of turnover of biomass are similar but that it is slightly lower in "*M. thiooxidans*".

It is not possible to calculate a theoretical Y_{max} for *Methylophaga* spp. growing under DMS-limitation as the dissimilation pathway remains, at this stage, unknown. Given that DMS contains twice as much carbon as methanol, one would anticipate higher Y during growth under DMS limitation than on methanol limitation – not necessarily a two-fold increase in Y_{max}, but certainly higher – however, the converse is true for *M. sulfidovorans*, which has an 8% lower Y_{max} on DMS *versus* methanol. "*M. thiooxidans*", on the other hand, has a 12.2% higher Y_{max} on DMS *versus* methanol. If it was assumed that approximately 48% dry biomass consists of carbon, this would equate to 4.42g carbon assimilated per mole DMS carbon (14.02g) by *M. sulfidovorans* or 6.91g by "*M. thiooxidans*". This indicates that a large proportion of DMS carbon ends up as CO_2 in order to meet the energy demends of the organisms during growth. It can be seen from these data that "*M. thiooxidans*" dissimilates less formaldehyde to CO_2 than *M. sulfidovorans*, indicating that it meets its energy demands *via* other means, thus coupling formaldehyde oxidation to growth more tightly than in *M. sulfidovorans*.

5.3.3 Determination of end-products of DMS metabolism

During growth on DMS, two end-products (with respect to sulfur) have been identified in previous studies. Thiosulfate has been demonstrated as the end-product of *M. sulfidovorans* (De Zwart *et al.* 1996), whereas *Hyphomicrobium* spp. have been shown to produce sulfate (De Bont *et al.* 1981; Borodina 2002). Studies of marine samples containing *Methylophaga* spp. using [^{35}S]-DMS have indicated the production of sulfate (Vila-Costa *et al.* 2006), however, such samples contained *Thiomicrospira* spp., which would be able to oxidise thiosulfate (along with other reduced sulfur compounds) to sulfate. It is worth noting that the analytical methods used by Vila-Costa *et al.* (*i.e.* that the production of a radioactive white precipitate upon the addition of barium chloride solution to samples incubated in the presence of [^{35}S]-DMS was diagnostic for sulfate) are invalid. As discussed in more detail in Chapter 3, at pH $\geq$ 2.0, Ba^{2+} will form a white precipitate with all reduced-sulfur species with the exception of thiocyanate.

In order to survey for reduced sulfur compounds, supernate obtained from a chemostat culture of "*M. thiooxidans*" under DMS-limitation ($D = 0.03h^{-1}$; $[DMS]_0 = 2mM$) was analysed by TLC against standards of thiosulfate and polythionates (Figure 5.3). Both thiosulfate and polythionate could be detected in the supernate.

In order to confirm which polythionate was produced by "*M. thiooxidans*" during growth on DMS, samples of supernate were subjected to analyses for thiosulfate and lower polythionates (Table 5.4). It was found that the dominant reduced sulfur species present in the supernate was tetrathionate, in contrast to the thiosulfate produced by the related DMS-oxidising *M. sulfidovorans*. A small quantity of thiosulfate was also found in the culture supernate, presumably an intermediate in the metabolism of DMS by "*M. thiooxidans*".

Species	$S_2O_3^{2-}$	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$	$S_6O_6^{2-}$	$S_7O_6^{2-}$
Concentration (mM)	0.3 (±0.1)	-	3.4 (±0.1)	-	-	-

Table 5.4. Concentrations of reduced sulfur compounds in the supernate of a culture of "*Methylophaga thiooxidans*" grown in a DMS-limited chemostat ($[DMS]_0 = 15mM$). "-" indicates compound below the detection limits of the assay. Figures in brackets are standard error of mean ($n = 3$).

Production of tetrathionate from an organosulfur compound has not been previously observed in any organism – as far as it is possible to tell from the literature. The observation that thiosulfate is seemingly produced as an intermediate during growth indicates that tetrathionate may be being formed from thiosulfate *via* thiosulfate dehydrogenase.

The products of DMS metabolism were also assessed in batch-cultures of *M. sulfidovorans* and *M. aminisulfidivorans* – in both cases, thiosulfate was confirmed as the end-product.

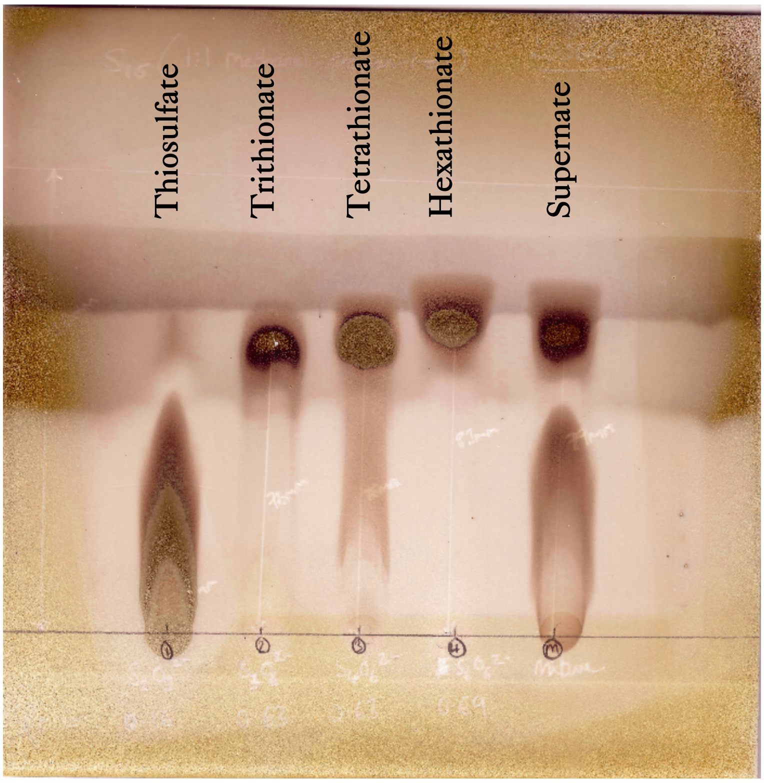

Figure 5.3. Thin-layer chromatogram of supernate obtained from a DMS-grown "*M. thiooxidans*" culture against reduced sulfur species standards, illustrating the presence of both thiosulfate and at least one polythionate in the supernate.

5.3.4 Addition of thiosulfate and polythionates to a DMS-limited chemostat

The effects of reduced inorganic sulfur compounds on "*M. thiooxidans*" grown under DMS-limitation were assessed. A steady-state was established on DMS ($D = 0.03h^{-1}$, $S_0 = 2mM$) and Y determined before each inorganic sulfur compound (thiosulfate, tri-, tetra-, penta-, hexathionate) was added to the vessel (in turn) to a final concentration of 0.2mM and 5 culture volume changes were allowed before redetermination of Y along with concentrations of DMS and inorganic sulfur compounds. A steady-state was re-established on DMS before the next inorganic sulfur compound was added to the chemostat. Table 5.5 shows Y and concentrations of inorganic sulfur compounds in the chemostat at steady-state. It can be seen that polythionates had no effect on Y and were not degraded by the organism. Thiosulfate, on the other hand, was consumed rapidly, causing an immediate perturbation of the steady-state with a drop in dissolved-oxygen concentration of the culture, with an accompanying increase in Y from 2.88g dry biomass per moles DMS to 3.19g (1.44g to 1.60g per mole of DMS carbon).

In order to assess the effects of thiosulfate on Y_{MAX} and m_{S}, steady-states were established between $D = 0.007\mathrm{h}^{-1}$ and $0.07\mathrm{h}^{-1}$ on DMS (S_0 = 2mM) with and without the addition of 0.2mM thiosulfate. At each steady-state, concentrations of thiosulfate and tetrathionate were measured, along with Y. The increase in Y_{max} per mole of thiosulfate oxidised was from 14.4g dry biomass per mole DMS to 15.7g as determined from a double-reciprocal plot. Whilst Y_{max} was found to increase, m_{S} remained constant at 8mmol DMS $\mathrm{g}^{-1}\cdot\mathrm{h}$, as determined from a plot of q *versus* D. This increase in Y_{max} without change in m_{S} is consistant with the data of Mason (1978) in *Pseudomonas* spp. and *Aeromonas* spp. in which Y_{max} and μ_{max} were stimulated by the addition of thiosulfate to cultures, resulting in the formation of tetrathionate, but without change in m_{S}.

Reduced sulfur compound added (200μM)	Y (g mol^{-1})	Concentration of reduced sulfur compounds at steady-state (μM)				
		$S_2O_3^{2-}$	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$	$S_6O_6^{2-}$
None	2.91	-	-	491 (±5)	-	-
Thiosulfate	3.19	-	-	593 (±3)	-	-
Trithionate	2.87	-	195 (±9)	486 (±7)	-	-
Tetrathionate	2.85	-	-	686 (±2)	-	-
Pentathionate	2.92	-	-	502 (±3)	190 (±4)	-
Hexathionate	2.86	-	-	488 (±2)	-	197 (±1)

Table 5.5. Concentrations of reduced sulfur compounds produced or consumed during growth of "*M. thiooxidans*" under DMS-limitation (D=0.03h^{-1}; [DMS]$_0$=2mM) with supplementary inorganic sulfur compounds added. "-" indicates that if the compound was present, it was below the detection limits of the assay. Figures in brackets are standard error of mean (n = 3).

5.3.5 Addition of thiosulfate and polythionates to a methanol-limited chemostat

To assess the effects of reduced sulfur compounds on growth under methanol-limitation, the experiment outlined in Section 5.3.4 was repeated using a steady-state established on methanol in place of DMS (D = 0.03h^{-1}, S_0 = 25mM) and inorganic sulfur compounds at a final concentration of 2.5mM. Once again, polythionates were shown to have no effect on Y; however, thiosulfate caused an immediate perturbation of the steady-state with a drop in dissolved-oxygen concentration of the culture. Table 5.6 shows Y and concentrations of inorganic sulfur compounds in the chemostat at steady-state. It can be seen that similar effects were observed to those found upon the addition of inorganic sulfur compounds to DMS-limited chemostats. The increase in Y_{max} per mole of thiosulfate oxidised was from 16.5g dry biomass per mole methanol to 18.0g, as determined from a double-reciprocal plot. Whilst Y_{max} was found to increase, m_S

remained constant at 8mmol methanol $g^{-1} \cdot h$, as determined from a plot of *q versus D*, consistant with the data obtained during the addition of thiosulfate to DMS-limited chemostats.

Reduced sulfur compound added (2.5mM)	*Y* ($gmol^{-1}$)	Concentration of reduced sulfur compounds at steady-state (mM)				
		$S_2O_3^{2-}$	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$	$S_6O_6^{2-}$
None	3.12	-	-	-	-	-
Thiosulfate	3.75	-	-	1.3 (±0. 1)	-	-
Trithionate	3.10	-	2.4 (±0.4)	-	-	-
Tetrathionate	3.12	-	-	2.7 (±0.1)	-	-
Pentathionate	3.15	-	-	-	2.6 (±0.1)	-
Hexathionate	3.13	-	-	-	-	2.2 (±0.2)

Table 5.6. Concentrations of reduced sulfur compounds produced or consumed during growth of "*M. thiooxidans*" under methanol-limitation (D=0.03h^{-1} S_0=25mM) with supplementary inorganic sulfur compounds added. "-" indicates that if the compound was present, it was below the detection limits of the assay. Figures in brackets are standard error (*n* = 3).

The rapid consumption of thiosulfate by a methanol-grown culture would appear to indicate that the enzymes responsible for thiosulfate oxidation are expressed constitutively during growth on methanol in the absence of thiosulfate. Owing to the stoichiometric conversion of thiosulfate to tetrathionate by "*M. thiooxidans*", it was felt that thiosulfate dehydrogenase[14] (EC 1.8.2.2) was likely to be the enzyme responsible. Cells of "*M. thiooxidans*" grown in batch-culture on thiophene-3-carboxylate, fructose or monomethylamine were found to oxidise thiosulfate and

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[14] Variously termed thiosulfate-oxidising enzyme, tetrathionate synthase and thiosulfate:ferricytochrome *c* oxidoreductase.

had thiosulfate dehydrogenase activity after no pre-exposure to thiosulfate (data not shown).

5.3.6 Addition of methanol to a DMS-limited chemostat

The stimulation of bacterial DMS- and sulfide- oxidation by the addition of methanol to mixed cultures, particularly in biofilters, has been previously observed (Jin *et al.* 2007; Zhang *et al.* 2007). Methanol was added to a DMS-limited chemostat ($D = 0.03h^{-1}$, $DMS_0 = 2mM$) to a final concentration of 2mM. As with thiosulfate oxidation, an immediate effect on the steady-state was observed, with a rapid drop in dissolved oxygen concentration and an increase in Y. As "*M. thiooxidans*" is known (Schäfer 2007) to express methanol dehydrogenase during growth on DMS, this is hardly surprising. Y increased from 2.91 to 3.30g per mole, indicating carbon from methanol was being assimilated to biomass. The amount of carbon assimilated from methanol was interestingly less than that expected, based on the Y_{max} observed during growth on methanol alone. In order to explain this, it is important to consider the internal limitations of the organism during growth. It is anticipated that *Methylophaga* spp. are ATP-limited during growth, as with most methylotrophic *Bacteria* that use the RuMP pathway (Anthony 1982). During growth on methanol, *Methylophaga* spp. fit the criteria for ATP-limited growth (Anthony 1982) in that NADH-dependent hydroxylations are not present and that oxidation of formaldehyde to CO_2 yields 2 moles NADH per mole of formaldehyde. During growth on DMS, no NADH-dependent reactions occur in the methyltransferase pathway anticipated to be present in *Methylophaga* spp. and formaldehyde oxidation would be expected to produce the same yield of NADH as in growth on methanol. As the *Y* on DMS is lower than that on methanol, it is clear that growth on these substrates is not energetically equivalent. Some RuMP pathway methylotrophs (*e.g. Methylobacillus flagellatus* C2A1, previously "*Aminomonas aminovorus*") are known to be limited by carbon during growth on methanol or other C_1 compounds due to the low demand for NADH during growth, as would be the case with *Methylophaga* spp. growing on DMS, however, if it were truly the case that *Methylophaga* spp. were limited by carbon during growth, virtually no formaldehyde would be oxidised to CO_2 to produce ATP and any "missing" carbon that does not end up as biomass would be expected to have been lost due to "leaky metabolism" and would appear in the culture supernates in the form of a metabolic intermediate. In the chemostat studies with "*M. thiooxidans*" performed here, the majority of substrate carbon appears to be lost as CO_2, in that there is no measurable build-up of the expected metabolic intermediates (*e.g.* methanethiol, formaldehyde) in the culture medium and the concentration of CO_2 evolved by the cultures is generally high (data not shown), indicating that formaldehyde oxidation is probably not coupled to growth but to energy metabolism.

5.4 Oxidation of homologues of DMS and other VOSCs

The oxidation of DMS homologues and other compounds similar to DMS was assessed in CFE obtained from DMS-grown cells. 2mL aliquots of CFE (10mg protein mL^{-1}) in 20mM Tris-HCl pH 7.4 were incubated at 25°C in 25mL glass serum vials sealed with butyl rubber vaccine stoppers in the presence of compounds under test at a concentration of 500μM; concentration of the substrates was assessed at 30 minute intervals for 8 hours. No oxidation of DMSO,

$DMSO_2$, diethylsulfide, dipropylsulfide, dibutylsulfide, dipentylsulfide, dihexylsulfide, diphenylsulfide, allyl methyl sulfide or allyl isothiocyanate was observed.

5.5 The effect of thiol-binding agents on DMS and thiosulfate metabolism

Thiosulfate oxidation by *Bacteria* has been previously shown to be affected by thiol-binding agents, both negatively (in the case of iodoacetamide (IA), *p*-chloromercuribenzoate (CMB), dibromohydroxymercurifluorescein (Mercurochrome™) and mercuric chloride ($HgCl_2$)) and positively (in the case of *N*-ethyl maleimide (NEM); Trudinger 1965) in *H. neapolitanus.* The effect of thiol-binding agents on DMS and thiosulfate metabolism in "*M. thiooxidans*" was assessed using CFE prepared from cells obtained from a DMS-limited chemostat (D = 0.07h^{-1} S_0 = 4mM). CFE was diluted in 20mM Tris-HCl pH 7.4 to give a final concentration of 10mg protein mL^{-1} before supplementing to 100μM with IA, CMB, Mercurochrome™, $HgCl_2$ or NEM from 1mM stock solutions in MilliQ water (CMB solutions were prepared as previously described by Trudinger (1965)) and preincubating for 30 minutes on ice. Treated extracts (1mL) were sealed, in triplicate, in 2mL serum vials with PTFE vaccine stoppers before supplementing with either DMS (to 800μM) or thiosulfate (to 400μM) and incubating for 16 hours at 25°C. In the case of whole-cells, the same method was used with CFE replaced by washed cells resuspended in 20mM Tris-HCl pH 7.4 to give an equivalent concentration of 30mg dry biomass L^{-1}. After incubation, DMS, MT, formaldehyde, sulfide, sulfite, sulfur, sulfate, thiosulfate, trithionate and tetrathionate were determined.

5.5.1 DMS metabolism

DMS was totally consumed by extracts in the presence or absence of all thiol-binding agents assessed during the 16 hour incubation period. Incomplete oxidation of DMS was observed in the presence of all thiol-binding agents (with the exception of NEM) as far as thiosulfate, as can be seen in Table 5.7. In the presence of NEM, oxidation of DMS to tetrathionate was complete.

Condition	Amount of sulfur compound after 16h (μmol)							
	DMS	MT	HCHO	S^{2-}	S^0	SO_3^{2-}	$S_2O_3^{2-}$	$S_4O_6^{2-}$
H_2O + DMS	410 (± 3)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
CFE + DMS	6 (± 2)	0 (± 0)	0 (± 0)	1 (± 0)	1 (± 0)	0 (± 0)	0 (± 0)	96 (± 4)
CFE + DMS + IA	3(± 4)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	187 (± 9)	1 (± 0)
CFE + DMS + CMB	2 (± 3)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	191 (± 9)	1 (± 0)
CFE + DMS + $HgCl_2$	3 (± 2)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	188 (± 9)	3 (± 1)
CFE + DMS + Mercurochrome™	1 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	206 (± 4)	1 (± 0)
CFE + DMS + NEM	0 (± 1)	0 (± 0)	20 (± 10)	0 (± 0)	0 (± 0)	0 (± 0)	4 (± 2)	98 (± 4)
H_2O + $S_2O_3^{2-}$	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	195 (± 4)	0 (± 0)
CFE + $S_2O_3^{2-}$	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	1 (± 1)	105 (± 6)
CFE + $S_2O_3^{2-}$ + IA	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	204 (± 6)	12 (± 3)
CFE + $S_2O_3^{2-}$ + CMB	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	200 (± 8)	4 (± 3)
CFE + $S_2O_3^{2-}$ + $HgCl_2$	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	183 (± 4)	8 (± 4)
CFE + $S_2O_3^{2-}$+ Mercurochrome™	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	184 (± 5)	2 (± 2)
CFE + $S_2O_3^{2-}$ + NEM	0 (± 0)	0 (± 0)	0 (± 0)	2 (± 0)	0 (± 0)	0 (± 0)	1 (± 1)	94 (± 1)

Table 5.7. Effect of thiol-binding agents on DMS and thiosulfate oxidation in extracts of "*Methylophaga thiooxidans*". Values in brackets denote standard error of mean (*n* = 3).

5.5.2 Thiosulfate metabolism

Thiosulfate oxidation to tetrathionate was inhibited by IA, Mercurochrome™, $HgCl_2$ and CMB but not by NEM (see Table 5.7). A red colouration was observed in assay mixtures containing NEM and thiosulfate (Figure 5.4), which is similar to previously observed reactions between NEM and sulfur compounds (Gregory 1955; Benesch *et al.* 1956; Smyth *et al.* 1960; Frese & Gröschel-Stewart 1972); however, it has been previously found that, in such reactions, ≥95% of the sulfur compound remains unchanged (Trudinger 1965), suggested a catalytic degradation of NEM in the presence of thiosulfate occurring in assay mixtures, preventing inhibition of the thiosulfate dehydrogenase enzyme.

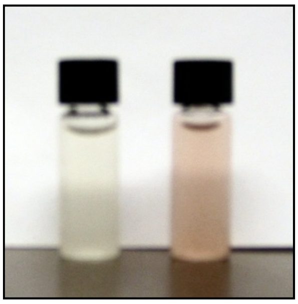

Figure 5.4. CFE incubated with thiosulfate in the presence of $HgCl_2$ (left) and NEM (right), showing the red colouration observed when NEM is incubated in the presence of thiosulfate.

Whilst CMB and $HgCl_2$ appear to inhibit thiosulfate oxidation, it is not clear whether they inhibit thiosulfate dehydrogenase or if they bind thiosulfate directly. Previous studies (Schellenberg & Schwarzenbach 1962) indicate that mercuric thiosulfate and thiosulfato-chloromercurobenzoate formation under the experimental conditions would be favourable, that these compounds are highly stable and thus effectively, thiosulfate can be considered to have been removed from the experiment.

In previous studies concerning the effects of thiol-binding agents on thiosulfate oxidation in *H. neapolitanus*, *Allochromatium vinosum* (previously "*Chromatium* D"), *Acidithiobacillus ferrooxidans* (previously "*T. ferrooxidans*") and *P. versutus* (previously *Thiobacillus* A2; Trudinger 1965; Hurlbert 1968; Kelly & Tuovinen 1975), inhibition by thiol-binding agents has always been shown to occur *after* oxidation to tetrathionate, thus, thiosulfate dehydrogenase was not shown to be inhibited by thiol-binding agents. In "*M. thiooxidans*", thiosulfate dehydrogenase was inhibited by thiol-binding agents, though it is unclear as to whether CMB or $HgCl_2$ truly inhibited the enzyme or merely bound to thiosulfate and "removed" it from the reaction.

5.6 The effect of group V and VI anions on thiosulfate metabolism

It has been previously shown (Kelly 1965; Kelly *et al.* 1997) that thiosulfate oxidation in *H. neapolitanus* (previously "*Thiobacillus* C" or "*T. neapolitanus*") can be stimulated by chromate and inhibited by selenate, whilst other group V and VI anions (metavanadate, molybdate and tungstate) were found to have various effects on the oxidation of tetrathionate to sulfate. In order to assess the effects of group V and VI anions on the oxidation of thiosulfate in "*M. thiooxidans*", whole-cell experiments identical to those detailed in Section 5.5 were set up in the presence of 250μmol chromate (CrO_4^{2-}), selenate (SeO_4^{2-}), metavanadate (VO_4^{3-}), molybdate

(MoO_4^{2-}) or tungstate (WO_4^{2-}) [15] in the place of the thiol-binding agents. Concentrations of thiosulfate and tetrathionate post incubation for various experimental conditions are given in Figure 5.5.

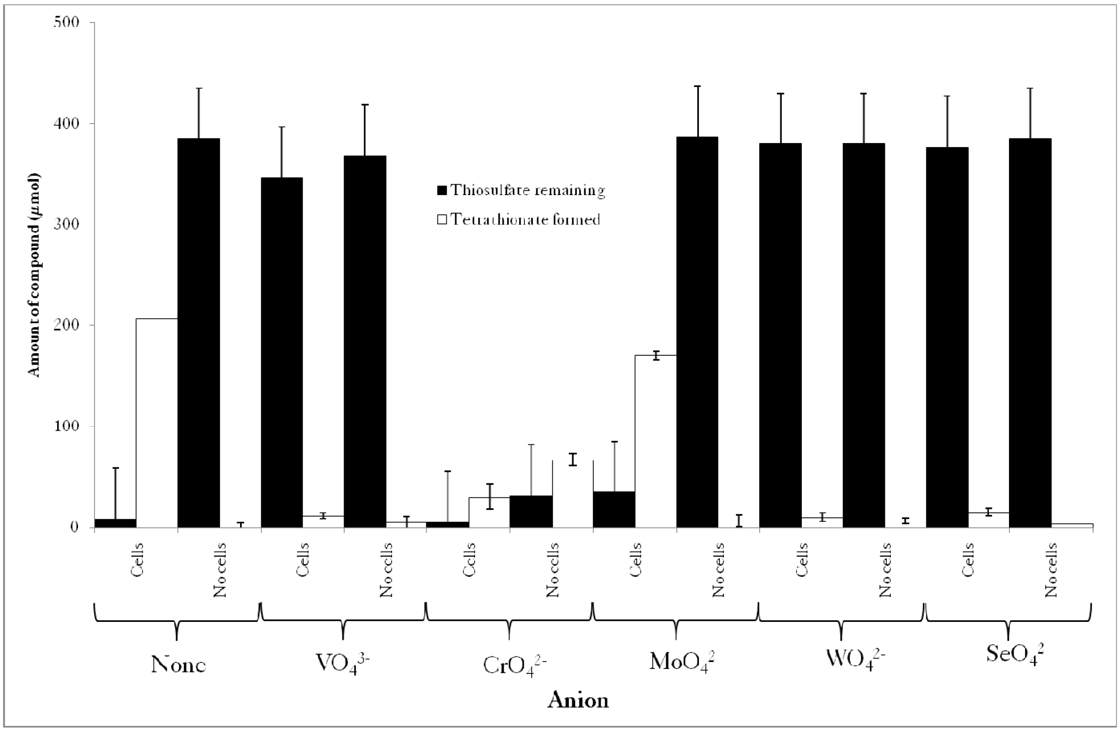

Figure 5.5. The effect of group V and VI anions on thiosulfate metabolism in "*Methylophaga thiooxidans*".

It can be seen from Figure 5.5 that the oxidation of thiosulfate to tetrathionate was inhibited by the presence of 250μmol tungstate, selenate or metavanadate. Oxidation was seemingly stimulated by chromate, as previously observed by Kelly *et al.* (1997); however, a large proportion of thiosulfate is oxidised in the absence of "*M. thiooxidans*", indicating that chemical oxidation by the chromate ion is the cause of the decrease in thiosulfate. Not all of the thiosulfate oxidised can be accounted for as tetrathionate, suggesting that tetrathionate is further chemically

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[15] As potassium chromate, sodium selenate, ammonium metavanadate, sodium molybdate and sodium tungstate. Controls with sodium, potassium or ammonium chloride were found to have no effect on thiosulfate oxidation.

oxidised by chromate, probably to sulfite and sulfate. Molybdate was observed to have little effect on the oxidation of thiosulfate. Selenate has previously been shown (Kelly *et al*. 1997) to inhibit thiosulfate oxidation in *H. neapolitanus* after the formation of tetrathionate – so without inhibiting thiosulfate dehydrogenase. In "*M. thioooxidans*", thiosulfate dehydrogenase is inhibited by selenate, indicating that, although the enzymes from the two *Bacteria* catalyse the same reaction, they may do so *via* different mechanisms. The stimulation of thiosulfate oxidation by molybdate observed in *H. neapolitanus* and *Paracoccus* spp. was not found in "*M. thiooxidans*" – with molybdate having no apparent effect on thiosulfate oxidation. Selenate (an analogue of sulfate) inhibited thiosulfate oxidation in *H. neapolitanus* after the formation of tetrathionate, so it is unsurprising that it had no effect on "*M. thiooxidans*". It is worth noting that the effect of selenate on *H. neapolitanus* has been described as being similar to that of thiol-binding agents (Kelly *et al*. 1997, Kelly & Tuovinen 1975), in that inhibition of thiosulfate oxidation occurred after the formation of tetrathionate. In "*M. thiooxidans*", thiol-binding agents inhibit the oxidation of thiosulfate to tetrathionate, indicating, again, that the thiosulfate dehydrogenase enzyme in "*M. thiooxidans*" is distinct from that in *Halothiobacillus* spp.

5.7 Coupling of thiosulfate oxidation to ATP synthesis

Since it has been demonstrated that thiosulfate oxidation to tetrathionate by "*M. thiooxidans*" results in an increase in Y_{max} in the chemostat under both DMS- and methanol-limitation, it would be assumed that electrons released during the oxidation are fed into the respiratory chain and result in the synthesis of ATP. In order to quantify ATP production from thiosulfate oxidation, "*M. thiooxidans*" cells were harvested from a DMS-limited chemostat ($D = 0.03h^{-1}$ $S_0 = 15mM$), washed in 20mM Tris-HCl pH 7.4 and resuspended in the same buffer to give an equivalent concentration of 30mg dry biomass L^{-1}. 10mL of the resuspended cells were placed in 25mL glass serum vials sealed with butyl rubber vaccine stoppers and were pre-incubated at 25°C for 30 minutes. Thiosulfate was added to give a final concentration of 1mM and suspensions were incubated, with shaking, at 25°C. Samples were removed at 15 second intervals for determination of ATP. The amount of ATP present per mg dry biomass over time is given in Figure 5.6.

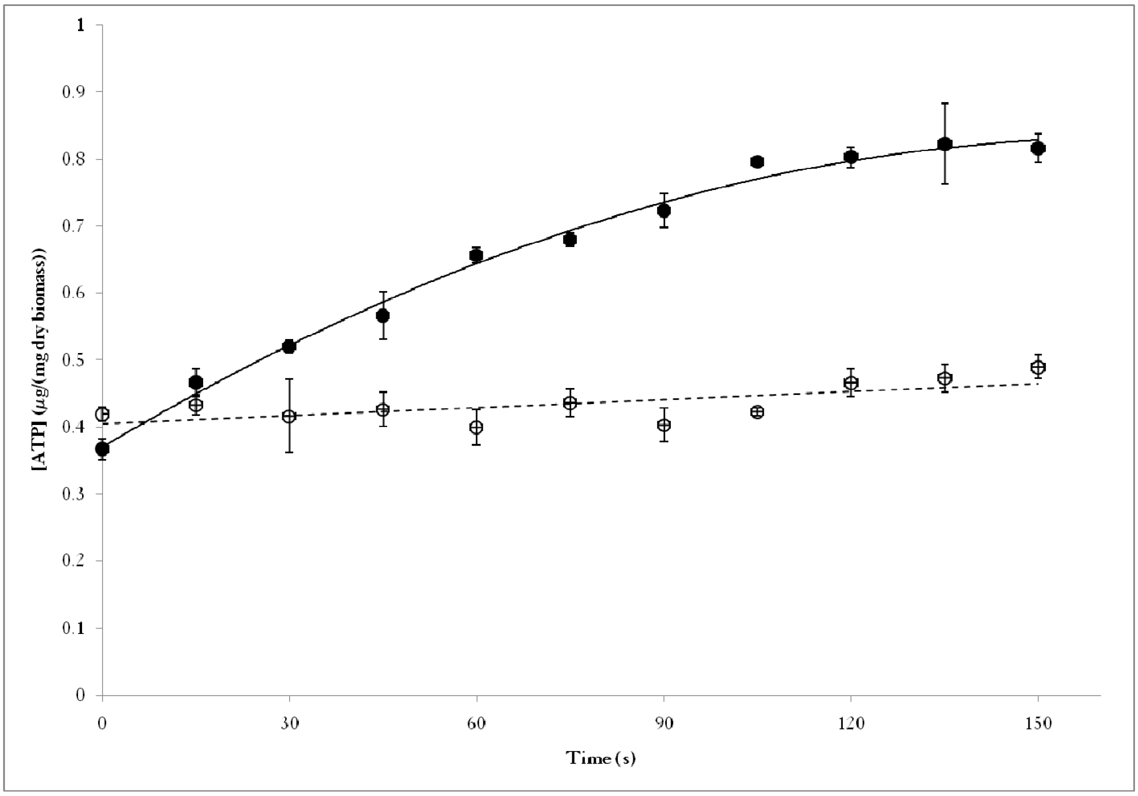

Figure 5.6. Formation of ATP during thiosulfate oxidation in whole-cells of "*Methylophaga thiooxidans*". Filled circles/solid line: cells incubated with thiosulfate; hollow circles/broken line: control cell incubation without thiosulfate. Error bars give standard error of mean ($n = 7$).

It can be seen from Figure 5.6 that ATP formation commences immediately upon the addition of thiosulfate to cells of "*M. thiooxidans*" with 0.13mg ATP (mg dry biomass)$^{-1}$ formed per mole of thiosulfate oxidised to tetrathionate. By comparison, *H. neapolitanus* produces 0.26mg ATP (mg dry biomass)$^{-1}$ per mole of thiosulfate oxidised to sulfate (Kelly & Syrett 1963, 1964) – an oxidation that takes place *via* tetrathionate. In the S_4I pathway of thiosulfate oxidation in *H. neapolitanus* (see Chapter 1), 20 protons and 16 electrons are passed on to the electron transport chain during tetrathionate oxidation, whereas the oxidation of thiosulfate to tetrathionate passes on just 2 electrons and 2 protons. If thiosulfate to tetrathionate oxidation in "*M. thiooxidans*" were equivalent to that of *H. neapolitanus*, half of the ATP produced during thiosulfate oxidation in *H. neapolitanus* would apparently be produced from 2 electrons and 2 protons, whereas the rest would come from 20 protons and 16 electrons, which would be highly unlikely. It is more likely that the initial thiosulfate oxidation in *H. neapolitanus* is less tightly coupled to ATP synthesis than in "*M. thiooxidans*", given the disproportionate amounts of ATP formed during the oxidation in the two organisms. It should of course be noted that this method of measuring ATP formation does not take into account cellular processes which are using ATP (*e.g.* chemotaxis) and the total amount "formed" is, of course, less the amount required for cellular maintainance. The m_S of *H. neapolitanus* growing on thiosulfate is 21.8mmol thiosulfate $g^{-1}\cdot h$ (Hempfling & Vishniac 1967, NB: this value was not obtained using the same strain of *H. neapolitanus* used by Kelly & Syrett but provides a good approximation of the maintainance requirements of the species) which is very high compared to that of "*M. thiooxidans*" growing on DMS – 8mmol DMS $g^{-1}\cdot h$. In short, the maintainance requirements of *H. neapolitanus* are much higher than that of "*M. thiooxidans*", suggesting that the values for ATP formation in *H. neapolitanus* given by Kelly & Syrett (1963, 1964) are much lower than the actual amount of ATP formed per mole of thiosulfate, whereas those from "*M. thiooxidans*" are certainly lower than the actual amount formed, but not by as great a margin. As such, the amount of ATP generated by thiosulfate dehydrogenase in "*M. thiooxidans*" might be equivalent to that in *H. neapolitanus*.

Since thiosulfate oxidation generates 2 moles of electrons per mole of tetrathionate formed, along with 0.13mg ATP (mg dry biomass)$^{-1}$, a $P/2e^-$ ratio of 0.13 could be calculated for "*M. thiooxidans*", however, this further demonstrates the fact that more than 0.13mg ATP must be formed during the thiosulfate oxidation as cytochrome *c* (to which thiosulfate dehydrogenase is probably coupled) can couple only with $P/2e^-$ ratios of 1 or 2.

In order to study the effect of uncoupling and pseudo-uncoupling agents on the production of ATP during thiosulfate oxidation by "*M. thiooxidans*", an experiment identical to that in Section 5.6 were set up with the inclusion of 1μmol of an uncoupling (DNP, CCCP or FCCP) or pseudo-uncoupling agent (picric acid) prior to the preincubation step (Kelly & Syrett 1966; Kessler *et al.* 1976). A positive control in which ATP synthesis was inhibited by the ATPase inhibitor DCCD (1μmol) was used. In the presence of picric acid, DNP, CCCP or FCCP, no thiosulfate-dependent ATP production was observed – demonstrating that ATP synthesis can be uncoupled from thiosulfate oxidation using routine uncoupling agents.

5.8 Enzymology of DMS oxidation

Enzymes known to be associated with C_1 and sulfur-compound metabolism were assayed in CFEs obtained from cells grown under methanol and DMS limitation (S_0 = 30mM or 15mM, respectively, D = 0.03h^{-1}). Specific enzyme activities are given in Table 5.8.

It can be seen from Table 5.8 that DMS monooxygenase activity is absent from "*M. thiooxidans*" during DMS-limited growth, whereas DMS "methyltransferase" activity is present. Methanol dehydrogenase activity is present during growth on both substrates tested, though it is present at slightly lower levels in DMS-grown cells. Methanethiol oxidase is present in DMS-grown cells and the catalase activity in these cells is higher than in methanol-grown cells – presumably due to the presence of an additional oxidase during growth on DMS compared to methanol.

Thiosulfate dehydrogenase activity is, as discussed previously (Section 5.3) present in DMS- and methanol-grown extracts. There is an approximately 2.5-fold increase in thiosulfate dehydrogenase activity in DMS-grown *versus* methanol-grown extracts.

In terms of formaldehyde assimilation, hydroxypyruvate reductase was absent, indicating the organism does not use the serine cycle, whereas 3-hexulose-6-

phosphate synthase was present, indicting the RuMP pathway was used. Fructose-1,6-bisphosphate aldolase activity was absent whereas KDPG aldolase activity was present – indicating that the KDPG-variant RuMP pathway was used for carbon assimilation, as is the case with all other *Methylophaga* spp.

In terms of enzymes of sulfur-metabolism, most were absent (Table 5.8). Two enzymes oxidising sulfide were induced during growth on DMS – the dehydrogenase, which produces elemental sulfur, and the oxygenase, which produces sulfite. Sulfur dioxygenase activity was absent, indicating that the progression from elemental sulfur is either chemical or *via* an unknown enzyme. APS reductase (used in sulfate assimilation) was found to be present though rhodanese activity was absent.

Enzyme assayed	EC	Specific activity (nmol min^{-1}(mg protein)$^{-1}$) of extracts obtained from cells grown on:	
		Methanol	DMS
Methanol dehydrogenase	1.1.1.244	782 (±4)	631 (±2)
Formaldehyde dehydrogenase (PMS)	1.2.1.46	186 (±1)	179 (±1)
Formaldehyde dehydrogenase (NAD^+)	1.2.1.1	0 (±0)	0 (±0)
Formate dehydrogenase	1.2.1.2	186 (±2)	179 (±1)
DMS monooxygenase	1.14.14.-	0 (±0)	2 (±0)
DMS methyltransferase/demethylase	2.1.1.-	0 (±0)	380 (±4)
DMS dehydrogenase	-	0 (±0)	0 (±0)
MT oxidase	1.8.3.4	0 (±0)	485 (±7)
DMSO reductase	-	0 (±0)	0 (±0)
$DMSO_2$ reductase	-	0 (±0)	0 (±0)
DMDS reductase	-	0 (±0)	0 (±0)
Sulfur dioxygenase	1.13.11.18	0 (±0)	0 (±0)
Sulfide dehydrogenase	-	0 (±0)	94 (±0)
Sulfide oxygenase	-	0 (±0)	69 (±3)
Sulfite dehydrogenase	1.8.2.1	0 (±0)	0 (±0)
Sulfite reductase (MV)	1.8.1.2	0 (±0)	0 (±0)
Thiosulfate dehydrogenase (FCN)	1.8.2.2	90 (±5)	216 (±8)
MSA monooxygenase	1.14.14.-	0 (±0)	0 (±0)
Alkanesulfonate monooxygenase[16]	1.14.14.5	0 (±0)	0 (±0)
CS_2 monooxygenase	-	0 (±0)	0 (±0)
Trithionate hydrolase	3.12.1.1	0 (±0)	0 (±0)
Rhodanese	2.8.1.1	0 (±0)	0 (±0)
Thiocyanate hydrolase	3.5.5.8	0 (±0)	0 (±0)
Thiosulfate reductase (GSH)	2.8.1.3	0 (±0)	0 (±0)
Thiosulfate reductase (DTT)	2.8.1.5	0 (±0)	0 (±0)
APS reductase	1.8.99.2	64 (±2)	72 (±7)
Catalase	1.11.1.6	295 (±9)	869 (±4)
NTA monooxygenase	1.14.14.-	0 (±0)	0 (±0)
3-Hexulose-6-phosphate synthase	4.1.2.-	495 (±2)	510 (±2)
Hydroxypyruvate reductase	1.1.1.81	0 (±0)	0 (±0)
Fructose-1,6-bisphosphate aldolase	4.1.2.13	0 (±0)	0 (±0)
KDPG aldolase	4.1.2.14	253 (±3)	270 (±1)

Table 5.8. Specific activities of enzymes in "*M. thiooxidans*" cell-free extracts prepared from cells obtained from DMS- or methanol-limited chemostats. Figures in brackets indicate standard error of mean ($n = 7$).

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[16] Activity given for hexanesulfonate.

5.9 Evidence for a DMS demethylase

During growth of "*M. thiooxidans*" in batch culture in sealed flasks, it was observed that, in old cultures (presumably oxygen-limited), MT built up but DMS oxidation continued to occur. This would appear to indicate that DMS oxidation is an oxygen-independent process in this strain. DMS "methyltransferase" enzyme was assayed in terms of the oxygen-independent oxidation of DMS and activities of 380nmol DMS oxidised minute^{-1} (mg protein)$^{-1}$ were found in "*M. thiooxidans*" CFE obtained from cells grown in a DMS-limited chemostat (Table 5.8).

The term "methyltransferase" refers to an enzyme that transfers a methyl- group *onto* a compound, rather than from it. In the case of the putative DMS "methyltransferase", a methyl- group is transferred from DMS onto an unknown carrier – thus, the enzyme should be more correctly termed DMS demethylase. Previous studies have suggested that a corrinoid-type cofactor is involved in this transfer process; however, there is no experimental evidence for this (Visscher & Taylor 1993a, b). For the formation of corrinoid cofactors, cobalt is a requirement of growth. The MAMS medium used for the cultivation of "*M. thiooxidans*" contains 3.22μM Co^{2+} in addition to 737nM cyanocobalamin (*i.e.* 737nM cobalt). Previous studies (Florencio *et al.* 1994) into the cobalt requirements of methanogenic *Archaea* which use corrinoid-dependent methyltransferases during growth suggest that growth can occur with Co^{2+} concentrations in the growth medium as low as 1.7nM, thus, the concentration of Co^{2+} in MAMS should be high enough to support corrinoid-dependent growth. *Methylophaga* spp. are usually described as having a requirement for "vitamin B_{12}" (usually supplied in the form of cyanocobalamin) during growth; however, strains of *M. marina* (KM3 and KM5, Li TD *et al.* 2007) have been isolated which do not have this requirement. It should be noted that media used in the cultivation of "B_{12}-requiring" *Methylophaga* spp. generally do not contain any cobalt, other than that bound as cyanocobalamin (Kim *et al.* 2007), so the true growth requirement of the genus may be cobalt, rather than B_{12}.

Known inhibitors of corrinoid-dependent enzymes include various alkyl and aryl iodides which are thought to inhibit the enzymes by alkylating the corrinoid cofactors (Brot & Weissbach 1965). These alkylations are reversed by strong light and so inhibition experiments were conducted in the dark. CFEs (diluted to contain 10mg protein mL^{-1}) prepared from cells of "*M. thiooxidans*" obtained from a DMS-limited chemostat were supplemented to $1\mu M$ with methyl iodide, ethyl iodide, *n*-propyl iodide, *n*-butyl iodide, iodobenzene, *n*-iodopropionate or IA according to Brot & Weissbach (1965). After pre-exposure to the iodine compounds on ice for 10 minutes in the dark, DMS demethylase activity was measured compared to a control (*i.e.* CFE supplemented with water in place of iodide solution and incubated on ice in the dark for 10 minutes). Activities relative to those of the control are given in Table 5.9. It can be seen that DMS demethylase was inhibited most strongly by *n*-iodopropionate, followed by *n*-butyl iodide. Total inhibition was not observed with any of the inhibitors, though only one concentration was used (1mM for 10mg protein mL^{-1}). In Brot & Weissbach's study, complete inhibition was observed with most alkyl iodides assayed at the specified concentration; however, since this was performed with purified protein rather than a CFE, non-specific binding of the inhibitor to other proteins would not have occurred (as will have undoubtedly occurred here) and, as such, all the inhibitor would have been able to act on the corrinoid cofactor of interest.

Inhibitor (1μM)	**Activity relative to the control (%)**
Methyl iodide	87 (±3)
Ethyl iodide	100 (±4)
n-Propyl iodide	92 (±6)
n-Butyl iodide	31 (±5)
Iodobenzene	100 (±2)
n-Iodopropionate	14 (±8)
Iodoacetate	80 (±5)

Table 5.9. The effect of corrinoid-dependent enzyme inhibitors on DMS demethylase activity in "*Methylophaga thiooxidans*". Activities are relative to that of an uninhibited control CFE (*i.e.* 195nmol DMS oxidised min^{-1} (mg protein)$^{-1}$). Figures in brackets are standard error of mean (n = 7).

In conclusion, an oxygen-independent mechanism is responsible for the initial oxidation of DMS to MT and there is some evidence (in terms of inhibition experiments) that a corrinoid cofactor may be involved, suggesting a demethylase. It is possible that a hydrolase could be at work here, yielding MT and methanol rather than MT and formaldehyde, which requires further investigation.

5.10 The role of methanol dehydrogenase in DMS metabolism

As mentioned in Section 5.9, it is possible that a DMS hydrolase could be responsible for the initial oxidation of DMS to MT, resulting in the formation of methanol. If this were the case, methanol dehydrogenase would be anticipated to be involved in the DMS oxidation pathway, oxidising methanol to formaldehyde. Previous studies with "*M. thiooxidans*" have identified the presence of the methanol dehydrogenase subunits in DMS-grown cells (Schäfer 2007) and the enzyme activity has also been shown in DMS-grown cells (Table 5.8). Since methanol dehydrogenase has been shown to be expressed during growth on C_1 compounds in various *Bacteria* even though it is not involved in the metabolism of these compounds (*e.g. Hyphomicrobium sulfonivorans* grown on fructose, Borodina 2002), it was not clear as to whether methanol dehydrogenase was involved in DMS metabolism in "*M. thiooxidans*". Whole cells of DMS- or methanol-grown "*M. thiooxidans*" were obtained from substrate-limited chemostats and were exposed, on ice, to 1mM cyclopropanol (Thompson *et al.* 1995) for 10 minutes before assaying DMS (0.5mM) or methanol (1mM) oxidation in terms of oxygen uptake against cyclopropanol-free control. Oxidation rates are given in Table 5.10.

Condition	**Oxidation rate** [nmol O_2 min^{-1} (mg dry biomass^{-1})] **MeOH**	**DMS**
MeOH-grown cells	223 (±18)	0 (±0)
MeOH-grown cells + 10mM cyclopropanol	0 (±0)	0 (±0)
DMS-grown cells	186 (±5)	11 (±3)
DMS-grown cells + 10mM cyclopropanol	0 (±0)	12 (±4)
Boiled MeOH-grown cells	0 (±0)	0 (±0)
Boiled DMS-grown cells	0 (±0)	0 (±0)

Table 5.10. The effect of cyclopropanol on DMS or methanol oxidation in cells of "*Methylophaga thiooxidans*" grown on methanol or DMS. Figures in brackets are standard error of mean (*n* = 7).

It can be seen from Table 5.10 that, whilst cyclopropanol inhibits methanol oxidation in cells grown on DMS or methanol, it has no effect on DMS oxidation. It can therefore be concluded that methanol dehydrogenase is not necessary for

DMS oxidation in "*M. thiooxidans*" and that methanol is not an intermediate in the DMS oxidation pathway. As such, the corrinoid-based demethylase proposed in Section 5.9 is considered to be the principle enzyme of DMS metabolism in this strain.

5.11 Cytochrome involvement in thiosulfate oxidation

It has here been demonstrated that thiosulfate oxidation in "*M. thiooxidans*" is coupled to ATP formation and that the pair of electrons liberated from each pair of thiosulfate ions condensed to tetrathionate enters the respiratory chain at some point. In previous studies (Lu & Kelly 1988*a*, *b*; Kelly *et al.* 1993), cytochrome *c* has been shown to be the electron acceptor from thiosulfate dehydrogenase. In order to investigate the nature of any cytochromes reduced during thiosulfate oxidation, spectra of CFEs in the presence of thiosulfate and various inhibitors were prepared (Figure 5.7).

Figure 5.7.A and B show control oxidised (hexachloroiridite) and reduced (dithionite) spectra of CFEs. Figure 5.7.C shows the reduction of CFE using thiosulfate. Absorbance maxima in the presence of thiosulfate (Figure 5.7.C) can be observed at approximately 415nm, 525nm and 550nm, indicative of the presence of a reduced *c*-type cytochrome (Kelly *et al.* 1993). In order to elucidate whether the electron transfer from thiosulfate dehydrogenase was direct or indirect (*via* the quinone pool), inhibition experiments were carried out using antimycin A or myxothiazol, which both inhibit electron transfer from the quinone pool to cytochrome *c*. The three absorbance maxima seen without inhibitor were

still observed in the presence of either inhibitor (Figure 5.7.D and E), indicating that thiosulfate dehydrogenase transfers electrons directly to cytochrome c_{550}. This is in keeping with the enzyme assay for thiosulfate dehydrogenase in which ferricyanide is used as the cofactor – a chemical homologue of cytochrome *c*.

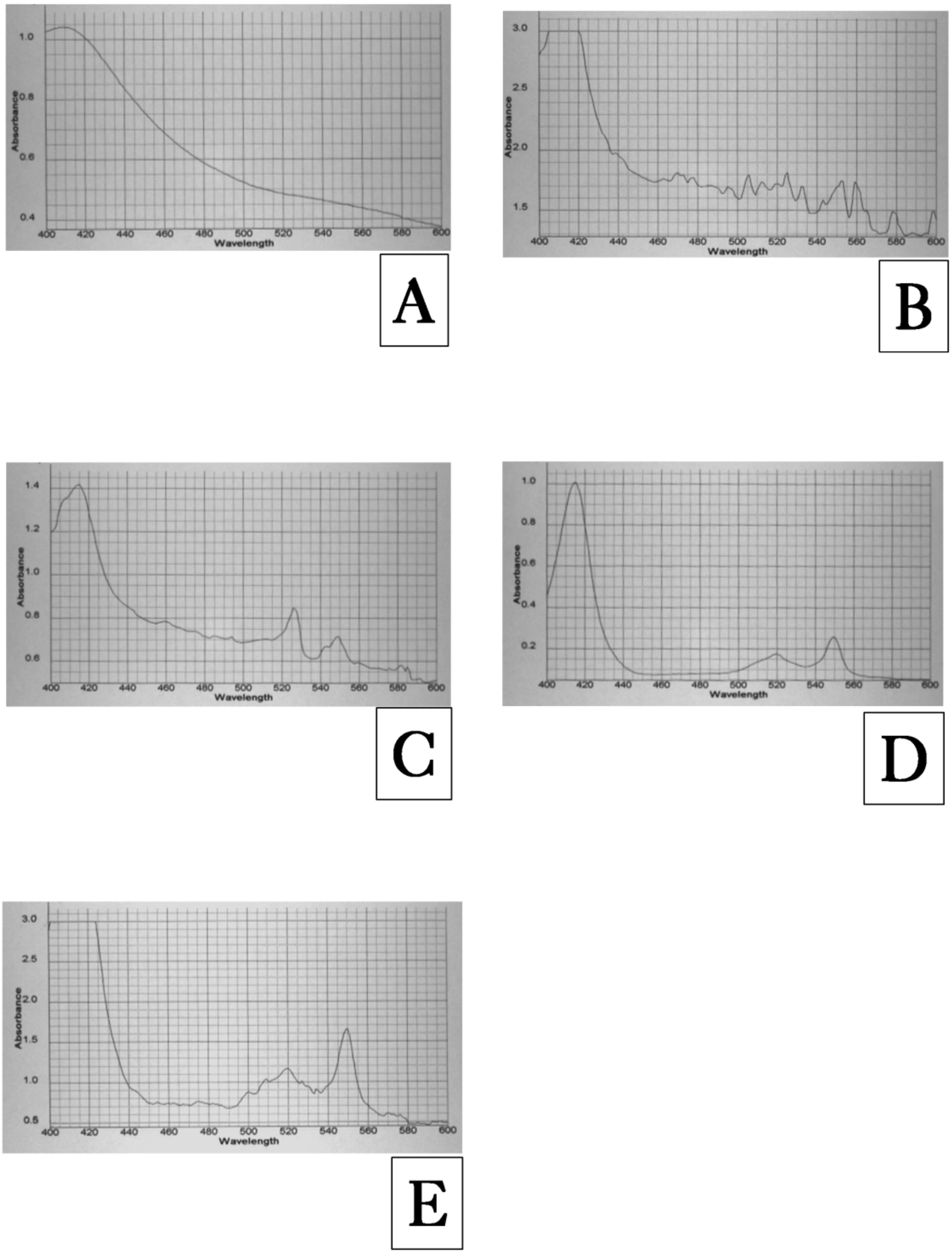

Figure 5.7. Spectra of cell-free extracts prepared from cells of "*Methylophaga thiooxidans*" obtained from a DMS-limited chemostat. A: oxidised extract, B: reduced extract, C: Extract + $S_2O_3^{2-}$, D: Extract + $S_2O_3^{2-}$ + Myxothiazol, E: Extract + $S_2O_3^{2-}$ + Antimycin A.

5.12 The role of the Suzuki & Silver reaction in thiosulfate formation

From enzyme assays of CFEs obtained from DMS-grown cells of "*M. thiooxidans*", it can be seen that sulfide dehydrogenase activity is present (Table 5.8), catalysing the oxidation of sulfide (HS^- in solution at physiological pH) to elemental sulfur (S^0). Observation of DMS-grown cells by dark-ground and phase-contrast light microscopy did not reveal the presence of intracellular sulfur granules within cells, as would be the case with many other *Bacteria* in which elemental sulfur is an intermediate. This suggests that, once formed, elemental sulfur is immediately converted into other species and does not build up within cells.

A second enzyme capable of sulfide oxidation has been shown to be active in "*M. thiooxidans*" grown on DMS – a sulfide oxygenase, catalysing the oxidation of HS^- to sulfite (SO_3^{2-}). Sulfite is a highly unstable species in solution, usually being spontaneously oxidised to sulfate (SO_4^{2-}). The absence of sulfate production by "*M. thiooxidans*" indicates that sulfite is rapidly converted by other means and does not become chemically oxidised to sulfate.

It has been previously shown in *T. thioparus* (Suzuki & Silver 1966) that thiosulfate can be formed from elemental sulfur and sulfite:

$$SO_3^{2-} + S^0 \rightarrow S_2O_3^{2-}$$

The reaction can be catalysed by the presence of glutathione in *T. thioparus* but the same process has been shown to occur under abiotic conditions in the absence of glutathione, indicating that it is purely a chemical process (Nedwell 1982).

Given that sulfite and elemental sulfur have both been shown to be formed enzymatically *via* sulfide oxygenase and sulfide dehydrogenase, respectively, and thiosulfate is also a known intermediate, it would seem highly likely that the Suzuki and Silver reaction is responsible for the formation of thiosulfate *in vivo* in "*M. thiooxidans*". In order to test this hypothesis, CFEs were incubated (as per previous inhibition experiments described in this chapter) under an atmosphere of argon in the presence or absence of 100μmol elemental sulfur in the form of *lac sulfuris* (as described in Chapter 2) or 100μmol sulfite, alone or in combination and sulfur, sulfite were determined after 16 hours incubation. Since it has been shown that Mercurochrome™ inhibits the oxidation of thiosulfate to tetrathionate in "*M. thiooxidans*", incubations were carried out with or without this inhibitor. Various biological and chemical controls were also incubated and monitored for activity. Concentrations of sulfur, sulfite and thiosulfate are given in Table 5.11.

Condition	Amount of species after 16h (μmol)		
	Sulfur	Sulfite	Thiosulfate
CFE	0 (±1)	0 (±0)	0 (±0)
CFE + Mercurochrome™	0 (±0)	0 (±0)	5 (±6)
CFE + S^0	88 (±13)	0 (±0)	3 (±3)
CFE + S^0 + Mercurochrome™	93 (±9)	0 (±0)	0 (±0)
CFE + SO_3^{2-}	0 (±0)	0 (±0)	0 (±0)
CFE + SO_3^{2-} + Mercurochrome™	0 (±0)	0 (±0)	0 (±0)
CFE + S^0 + SO_3^{2-}	4 (±3)	0 (±0)	14(±5)
CFE + S^0 + SO_3^{2-} + Mercurochrome™	0 (±0)	0 (±0)	94 (±2)
H_2O	0 (±0)	0 (±0)	0 (±0)
H_2O + Mercurochrome™	0 (±0)	0 (±0)	0 (±0)
H_2O + S^0	96 (±12)	0 (±0)	0 (±0)
H_2O + S^0 + Mercurochrome™	93 (±16)	0 (±0)	0 (±0)
H_2O + SO_3^{2-}	0 (±0)	0 (±0)	0 (±0)
H_2O + SO_3^{2-} + Mercurochrome™	0 (±0)	0 (±0)	0 (±0)
H_2O + S^0 + SO_3^{2-}	12 (±8)	0 (±0)	76 (±3)
H_2O + S^0 + SO_3^{2-} + Mercurochrome™	7 (±3)	0 (±0)	81 (±3)

Table 5.11. Oxidation of sulfur and sulfite to form thiosulfate in cell-free extracts of "*M. thiooxidans*" prepared from cells obtained from a DMS-limited chemostat or in water. Figures in brackets are standard error of mean (*n* = 3).

It can be seen from Table 5.11 that the formation of thiosulfate from sulfite and sulfur occurs in the presence and absence of CFE. In CFE, Mercurochrome™ is required in order to detect thiosulfate formation as thiosulfate dehydrogenase must be inhibited in order to avoid the immediate condensation of thiosulfate to tetrathionate (which is not observed in the water controls). Sulfite is seen to be fully oxidised in all experimental conditions – almost certainly due to chemical oxidation of unreacted sulfite to sulfate which is spontaneous in aqueous solutions in the presence of oxygen. The amount of thiosulfate formed from sulfite and sulfur in the water controls is slightly lower than in CFE samples, indicating that the reaction is probably catalysed by something present in the CFE, though given the small magnitude of the difference in thiosulfate formation, it would be anticipated that this is either a catalytic cofactor (*e.g.* glutathione) or a metal ion, rather than an enzyme.

5.13 The effects of inhibitors on DMS and thiosulfate metabolism

Previous studies into the metabolism of DMS implicated methyl *tert*-butyl ether (MTBE) and chloroform as inhibitors of the DMS monooxygenase and "methyltransferase" enzymes, respectively (Visscher & Taylor 1993a, b). 3-amino-1,2,4-triazole (Amitrole) has also been used in studies into DMS metabolism to inhibit the catalase used to reduce hydrogen peroxide formed by MT oxidase to water. It has been demonstrated (Chapter 4) that MTBE does not inhibit the pure DMS monooxygenase enzyme, but it does inhibit DMS oxidation in whole cells of *Hyphomicrobium sulfonivorans*. Previous studies with "*M. thiooxidans*" have shown that MTBE and chloroform have no effect on DMS oxidation (Schäfer 2007). It is not entirely clear from the literature as to where the notion that MTBE or chloroform would specifically inhibit DMS monooxygenase or "methyltransferase" came from. Chloroform is sometimes used to inhibit acetyl co-enzyme A transferases in methanogenic *Archaea* (Lovley & Klug 1982), so it is likely that it was incorrectly assumed to be a general inhibitor of methyltransferases. Since MTBE and chloroform are toxic to most *Bacteria*, their "inhibitory" effects on DMS metabolism are more likely to be toxic effects on cells in general, resulting in lower substrate oxidation rates.

Since it has already been shown that *n*-iodopropionate inhibits the DMS demethylase system in "*M. thiooxidans*" and Mercurochrome™ inhibits the thiosulfate dehydrogenase, the effects of other metabolic inhibitors were assessed. Diphenyleneiodonium chloride (DPI), arsenite (AsO_2^-), 2-(3-hydroxymercurio-2-methoxypropylcarbamoyl)phenoxyacetate (mersal), rotenone, ethyl vinyl sulfide (EVS) and 8-hydroxyquinoline *N*-oxide (HQNO) were assessed. Uncoupling and pseudo-uncoupling agents (FCCP, CCCP, DNP and picric acid) were reassessed to see if their action on preventing ATP synthesis during thiosulfate oxidation also prevented the oxidation itself from occuring. Identical experiments to the various metal anion and thiol-binding agent experiments already described were set up in the presence of the above inhibitors alongside controls. Inhibitors were assessed on CFE, whereas uncoupling agents were assessed on whole cells. Oxidation of DMS (400μmol) was monitored after 16h and suspected intermediates (*i.e.* MT, formaldehyde, sulfide, sulfite, elemental sulfur, thiosulfate and tetrathionate) were quantified. Quantities of intermediate found in each experimental condition are given in Table 5.12.

From Table 5.12 it can be seen that EVS, mersal and AsO_2^- inhibited DMS oxidation completely. EVS is a potent suicide inhibitor of *S*-adenosylmethionine:thioether *S*-methyltransferase (EC. 2.1.1.96), the mechanism of inhibition involving EVS acting as an alternative cofactor for this corrinoid-dependent enzyme, resulting in the formation of the methyl ethyl vinyl sulfonium anion, which is highly reactive and binds to amino acids around the active site of the enzyme (Warner & Hoffman 1996). It is probable that EVS could act as a substitute cofactor for the corrinoid-dependent DMS demethylase, resulting in the formation of MEVS and resulting in inhibition of the enzyme.

Since no oxidation of DMS to MT or HCHO appears to have occurred at all in the presence of EVS, it would appear that the site of inhibition is the DMS demethylase system. Mersal had similar effects to EVS, inhibiting metabolism at the point of DMS demethylase. Mersal is a hydrophilic thiol-binding agent, which presumably binds to the active site of the DMS demethylase. AsO_2^- is a potent inhibitor of thiol-dependent dehydrogenases and its mechanism of inhibition involves binding to thiols (*e.g.* glutathione), preventing them from acting as cofactors for enzymes. It would be expected, therefore, that AsO_2^- would inhibit after the initial oxidation to MT, by binding to the MT itself; however, it can be seen from Table 5.12 that this is not the case. AsO_2^- inhibits DMS oxidation completely, suggesting that it inhibits the DMS demethylase enzyme, rather than MT oxidase (in which case DMS would still be consumed but MT would build up). There is some evidence that corrinoid-dependent demethylases and methyltransferases possess vicinal thiol groups to which the corrinoid cofactor is associated (Hogenkamp 1968). The inhibition of corrinoid-dependent enzymes by AsO_2^- has been previously demonstrated and is thought to be due to the competition of corrinoid-binding sites in the enzyme by the AsO_2^- ion. Inhibition of DMS oxidation in "*M. thiooxidans*" extracts by AsO_2^- gives further evidence that the DMS demethylase is a corrinoid-dependent enzyme.

DPI inhibited DMS oxidation at the point of MT oxidase, leading to a build-up of MT. There is no associated build-up of any other intermediate, indicating that formaldehyde oxidation (from formaldehyde produced by the DMS demethylase system) proceeds as normal. DPI is a widely used inhibitor of flavoproteins and is known to inhibit many oxidase-type enzymes, though it has been shown not to specifically inhibit flavoproteins, but to phenylate a variety of enzyme cofactors. The association of DPI inhibition with oxidases in general appears to be due to the requirement of hydrogen peroxide to form a DPI· radical which then attacks positively charged regions of enzyme cofactors (O'Donnell *et al.* 1993). MT oxidase has been shown (Myronova, Boden & Schäfer, *unpublished data*) to be a pyrroloquinoline quinone (PQQ)-dependent enzyme, related to the copper-containing amine-oxidases (EC. 1.3.4.6). There is some evidence that DPI binds to PQQ irreversibly (Shiemke *et al.* 2004; Stites *et al.* 2006), thus suggesting that this binding could be behind the inhibition of MT oxidase by DPI.

Rotenone allowed DMS oxidation to proceed as far as thiosulfate formation but then inhibited the terminal oxidation step to tetrathionate. This is an unusual observation, since rotenone would be expected to inhibit electron transfer *via* the quinone pool, in the same way as antimycin A or myxothiazol; however, it has been shown (*cf.* 5.11) that antimycin A and myxothiazol do not inhibit electron transfer onto cyt c_{550} from thiosulfate dehydrogenase, thus suggesting that transfer is direct and does not go *via* the quinone pool. The most likely explanation for this is that rotenone blocks electron transfer from thiosulfate dehydrogenase to cyt c_{550} by blocking the cyt c binding-site on the dehydrogenase itself.

Condition	Amount of species after 16h (μmol)							
	DMS	MT	HCHO	S^{2-}	S^0	SO_3^{2-}	$S_2O_3^{2-}$	$S_4O_6^{2-}$
H_2O	393 (± 7)	0 (± 0)	1 (± 1)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
CFE	27 (± 1)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	92 (± 4)
CFE + DPI	202 (± 4)	208 (± 10)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
CFE + AsO_2^-	383 (± 3)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
CFE + Rotenone	29 (± 2)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	178 (± 4)	2 (± 1)
CFE + Mersal	370 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
CFE + HQNO	20 (± 0)	0 (± 0)	30 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	53 (± 4)
CFE + EVS	385 (± 8)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
Whole cells	80 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	5 (± 0)	65 (± 1)
Whole cells + DNP	140 (± 6)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	110 (± 4)	9 (± 1)
Whole cells + FCCP	150 (± 6)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	108 (± 2)	11 (± 2)
Whole cells + CCCP	150 (± 6)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	109 (± 3)	6 (± 1)
Whole cells + Picric acid	313 (± 2)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)	30 (± 3)	0 (± 0)

Table 5.12. Effect of inhibitors and uncoupling agents on DMS oxidation in whole-cells and extracts of "*Methylophaga thiooxidans*". Values in brackets denote standard error of mean ($n = 3$).

5.14 Summary

"*Methylophaga thiooxidans*" has been shown to grow on a limited range of carbon sources including methanol, DMS, methylated amines, substituted thiophenes, higher alkanesulfonates and fructose. Growth on DMS has been shown to be *via* a novel pathway, resulting in the formation of tetrathionate as the sulfur end-product.

A schematic showing the proposed pathway of DMS oxidation in "*Methylophaga thiooxidans*" is given in Figure 5.8, showing inhibitors of each step.

Tetrathionate has been shown to be formed from the oxidation of oxidation of thiosulfate by thiosulfate dehydrogenase. The thiosulfate dehydrogenase present in "*M. thiooxidans*" has been shown to be different from those of *Halothiobacillus* spp., *Thiobacillus* spp., *Allochromatium* spp. and *Acidithiobacillus* spp. in terms of its properties with respect to inhibition by thiol-binding agents and by group V and VI anions. The production of a tetrathionate from DMS it not only a novel product of DMS metabolism but it is worth noting that, as far as can be known from the literature, the production of any polythionate from an organosulfur compound is, thus far, undocumented.

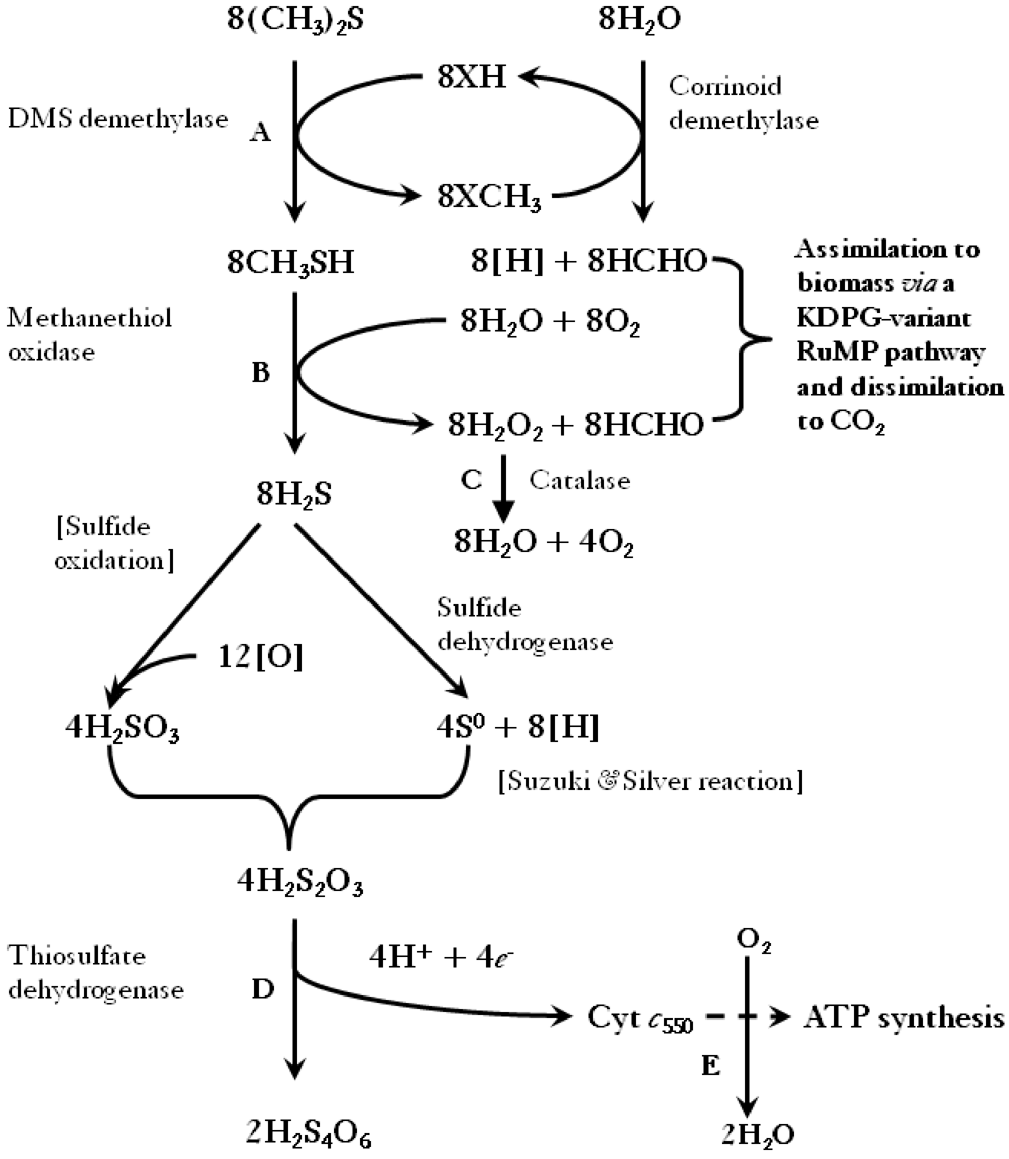

Figure 5.8. The proposed pathway of DMS metabolism in "*Methylophaga thiooxidans*", balanced with respect to respiratory oxygen. Ionic species are shown as conjugate acids for clarity. Inhibitors are indicated: A – *n*-iodopropionate, B – DPI chloride, C – Amitrole, D - Mercurochrome™, E – FCCP, CCCP, DNP.

The enzymes responsible for the oxidation of DMS through to formaldehyde are a putative corrinoid dependent DMS-demethylase and methanethiol oxidase. Thiosulfate is formed by a mixture of chemical and biological steps. Thiosulfate oxidation has been shown to be coupled to ATP production in "*M. thiooxidans*" with electrons liberated from the condensation of thiosulfate joining the respiratory chain at the level of cytochrome c_{550}. ATP production can be uncoupled from thiosulfate oxidation by standard uncoupling agents.

In terms of future work with "*M. thiooxidans*", priority is to purify the DMS demethylase/corrinoid demethylase complex. An attempt during the course of this study was made to purify the enzyme, which was found to be highly unstable during gel filtration experiments – probably due to the loss of a bound cofactor, such as the corrinoid itself. Once purified, peptide sequences could be obtained and the gene(s) determined from the genome of "*M. thiooxidans*". Southern blot analysis using the cloned gene(s) against other non-DMS monooxygenase-utilising DMS oxidising *Bacteria* could pave the way for the development of functional gene probes for molecular ecology studies.

5.15 Description of *Methylophaga thiooxidans* sp. nov

Methylophaga thiooxidans (thi.o. ox'i. dans. Greek noun *thion* sulfur; Modern Latin verb *oxido* to make acid, to oxidise; Modern Latin partial adjective *thiooxidans*, oxidising sulfur). Cells are straight rods that are around 0.6μm wide and around 1.8μm long. Cells occur singly, are motile by a single polar flagellum and multiply by binary fission. Colonies on MAMS agar incubated in an atmosphere containing methanol are flat and circular with an entire margin and a smooth surface. Colonies growing on methanol are 2-4mm in diameter after 48h growth. Vitamin B_{12} is not required for growth. Moderately halophilic. Catalase, oxidase and urease positive. Reduces nitrate to nitrite. Produces indole from tryptophan. Nitrate, ammonium, urea, monomethylamine, dimethylamine and trimethylamine (but not nitrite, cyanate, thiocyanate or dinitrogen) are used as nitrogen sources.

The species is chemolithoheterotrophic with thiosulfate stimulating growth whilst being oxidised to tetrathionate. Polythionates (S_3-S_6) do not support growth. Chemolithoheterotrophic growth is observed on dimethylsulfide and methanethiol with the sulfur moities being oxidised to tetrathionate whilst the carbon is assimilated to biomass.

A variety of compounds support heterotrophic growth: methanol, monomethylamine, dimethylamine, trimethylamine, fructose, thiophene-2-carboxylate, thiophene-3-carboxylate, thiophene-2-methylamine, *n*-caprylsulfonate, *n*-laurylsulfonate and *n*-myristylsulfonate. Growth was not observed on other sugars, amino acids, organic acids or on complex media. C_1 compounds are assimilated to biomass *via* the KDPG aldolase variant RuMP pathway.

The G+C content is 45.9mol% and the major ubiquinone is UQ-8. The type strain DMS010^T was isolated from pooled cultures of *Emiliania huxleyi*.

CHAPTER 6

ECOLOGY OF *Methylophaga* spp. & ENVIRONMENTAL CHEMISTRY OF PHYTOPLANKTON BLOOMS

6.1 Introduction

Aside from *Methylophaga* spp., marine *Bacteria* that are capable of growth on DMS are poorly described (see Chapter 1 for details of DMS oxidising *Bacteria* isolated and characterised to date). Since isolation-based studies (Schäfer 2007) have obtained pure cultures of *Methylophaga* spp. able to grow on DMS as a sole carbon source, it was felt necessary to investigate the diversity of marine *Bacteria* able to assimilate carbon from DMS using culture-independent methods.

VOSCs have been previously analysed during phytoplankton blooms and it is known that DMS is produced (Kwint & Kramer 1995); however, no studies have quantified inorganic sulfur species (such as thiosulfate or polythionates) under such conditions. Since "*Methylophaga thiooxidans*" has been shown to oxidise exogenous thiosulfate to tetrathionate during growth on DMS or methanol - both known to be produced during a phytoplankton bloom (Kwint & Kramer 1995; Heikes *et al.* 2002) – an upshift in the oxidation of marine thiosulfate to tetrathionate would be expected during a bloom situation.

6.2 Molecular ecology of DMS-oxidising marine *Bacteria* using stable-isotope probing

"*Methylophaga thiooxidans*" was originally isolated from a DMS enrichment culture inoculated with a non-axenic culture of *Emiliania huxlei* (Schäfer 2007). In order to assess the prevalence of "*M. thiooxidans*" in the marine environment, stable-isotope probing (SIP) methodology was employed.

The SIP methodology is explained in full in Chapter 2. Briefly, samples of seawater were supplemented with [$^{13}C_2$]-DMS and were incubated until DMS had been consumed. Biomass was harvested by filtration and DNA extracted. Isopycnic centrifugation was used to separate [$^{12}C_n$]-DNA from [$^{13}C_n$]-DNA. [$^{13}C_n$]-DNA was recovered and the polymerase chain reaction (PCR) used to amplify a region of the 16S rRNA (*rrs*) gene. Amplicons were separated by denaturing-gradient gel electrophoresis (DGGE) and sequences were obtained.

6.2.1 English Channel, UK, November 2005

The DGGE showing *Bacteria* which assimilated carbon from [$^{13}C_2$]-DMS is shown in Figure 6.1. PCR products were obtained from 4 DGGE bands from the fraction corresponding to the [$^{13}C_n$]-DNA fraction of DNA extracted from SIP incubations with [$^{13}C_2$]-DMS, the identities of which are given in Figure 6.2. It can be seen from Figure 6.2 that DGGE bands A – C represent 16S rRNA gene sequences clustered within the genus *Methylophaga* whereas band D fell within the genus *Alcanivorax*. Based on partial-sequences of the 16S rRNA gene, bands A – C appear to represent 16S rRNA genes with a high degree of identity to that of "*M. thiooxidans*", however, it is not possible to determine the exact phylogenetic position of such short sequences. Band D represented a 16S rRNA gene with a high degree of identity to that of *Alcanivorax venustensis*, which is not a species previously known to oxidise DMS. *Alcanivorax* spp. are known to use fatty acids as a source of carbon, so it is entirely possible that the uptake of ^{13}C observed here is not from DMS directly, but derived from lysed *Methylophaga* spp. which have assimilated carbon from [$^{13}C_2$]-DMS into their lipid membranes (Yakimov *et al.*

1998). Of course, it is possible that *Alcanivorax* spp. are capable of growth on DMS, though attempts at isolating them have failed (data not shown).

6.2.2 Phytoplankton bloom transect, English Channel, UK July 2006

A copy of the manuscript containing this study in full (Neufeld *et al.* 2008) can be found in the Appendices.

During a study into the prevalence of DMS-utilising *Bacteria* during a phytoplankton bloom (*i.e.* during a time at which the concentration of DMS in the water column is particularly high) undertaken in July 2006, *Methylophaga* spp. were found to be the only *Bacteria* which assimilated carbon from [$^{13}C_2$]-DMS. It should be noted, however, that 16S rRNA gene sequences obtained from [$^{13}C_2$]-DMS SIP experiments do not relate directly to "*M. thiooxidans*", though "*M. thioxidans*"-like populations (in Neufeld *et al.* (2008), "*M. thiooxidans*" is refered to as "*Methylophaga* sp. DMS010") were found in [^{13}C]-MMA and [$^{13}C_2$]-DMA SIP experiments in the same study.

It can be concluded from SIP experiments that, whilst *Methylophaga* spp. are the dominant *Bacteria* which take up carbon from [$^{13}C_2$]-DMS under the conditions of the SIP incubations, the organisms found during a phytoplankton bloom were not closely related to "*Methylophaga thiooxidans*". Whilst this is initially surprising, given that "*M. thiooxidans*" was isolated from a phytoplankton culture, the actual conditions of isolation explain why "*M. thiooxidans*" as we know it may not be directly detectable in the environment. Table 6.1 gives details of the *E. huxleyi* cultures (Schäfer 2007) which were pooled and used as innoculum for the DMS enrichment cultures from which *"M. thiooxidans"* was isolated.

E. huxleyi strain	Isolated from	Isolation date
CCMP371	Sargasso Sea	20th June 1987
CCMP373	Sargasso Sea	4th January 1960
CCMP379 ("92A")	English Channel	24th July 1957
CCMP1516	South Pacific	7th May 1991

Table 6.1. Isolation details of *Emiliania huxleyi* cultures used as inoculum for enrichment cultures from which *"Methylophaga thiooxidans"* was isolated (Schäfer 2007 and The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Boothbay Harbour, ME, USA) online catalogue, accessed 9th March 2009 http://ccmp.bigelow.org).

It can be seen from Table 6.1 that *E. huxleyi* cultures used for the isolation of *"M. thiooxidans"* varied in age from 13 to 47 years at the time they were used for isolation of the strain (2004). Given this time interval and the maintainance of the phytoplankton as a live culture, along with its associated *Bacteria*, it would be fair to conclude that from whichever sampling site *"M. thiooxidans"* actually originated, the organism would most definitely have evolved as a lab strain and one would not expect to find "*M. thiooxidans*" *sensu* DMS010 in the environment. The English Channel, Sargasso Sea and South Pacific are very different environments in terms of temperature, nutrient availability, light and various other selection pressures. Baas Becking (1934) stated that "*alles is overal:* maar *het milieu selecteert*" ("everything is everywhere: *but* the environment selects"), which is taken to mean that environmental pressures select for and against various organisms in a given environment, but, at some point in the distant past, all (micro)organisms were probably present there at some level. This theory of course applies to mixed cultures in the laboratory as well as to the outside environment and it can easily be seen how *Methylophaga* spp. living within an *E. huxleyi* culture in the lab would not be subject to some of the negative pressures of the marine environment (such as ultraviolet light, phages, antimicrobial agents, mechanical sheering from waves *etc*.) and would be subject to different positive pressures (such as a constant supply of DMS from *E. huxleyi* cells). Additionally, different negative pressures exist in a laboratory culture (*e.g.* "bottle effect", pH changes, limited supplies of certain trace elements) that do not apply in the marine environment. As such, "*M. thiooxidans*" could be considered to be a lab-optimised descendant of a wild-type *Methylophaga* sp. that may still be present in the environment. It is worth noting that 16S rRNA gene sequences more similar to that of "*M. thiooxidans*" were found in the November 2005 SIP, in which 60μM [$^{13}C_2$]-DMS was used, than in the July 2006 SIP in which 250μM [$^{13}C_2$]-DMS was used. This could indicate that "*M. thiooxidans*"-like organisms have a particularly high-affinity for DMS and are able to assimilate it to biomass at low concentrations. In the July 2006 experiments using higher concentrations of DMS and with an already induced population of DMS-oxidising *Bacteria* in the environment assayed (*i.e.* a phytoplankton bloom), it is possible that "*M. thiooxidans*"-type organisms were selected against under the conditions the SIP experiments were conducted in.

Even though "*M. thiooxidans*" itself cannot be found during SIP experiments or in terms of sequence data from the Global Ocean Sampling project (an 86% identity sequence to the "*M. thiooxidans*" 16S rRNA gene can be found in sequence EK519039 by searching with the BLASTn algorithm), the question still remains:

are there *Bacteria* present in the marine environment that can produce tetrathionate from DMS?

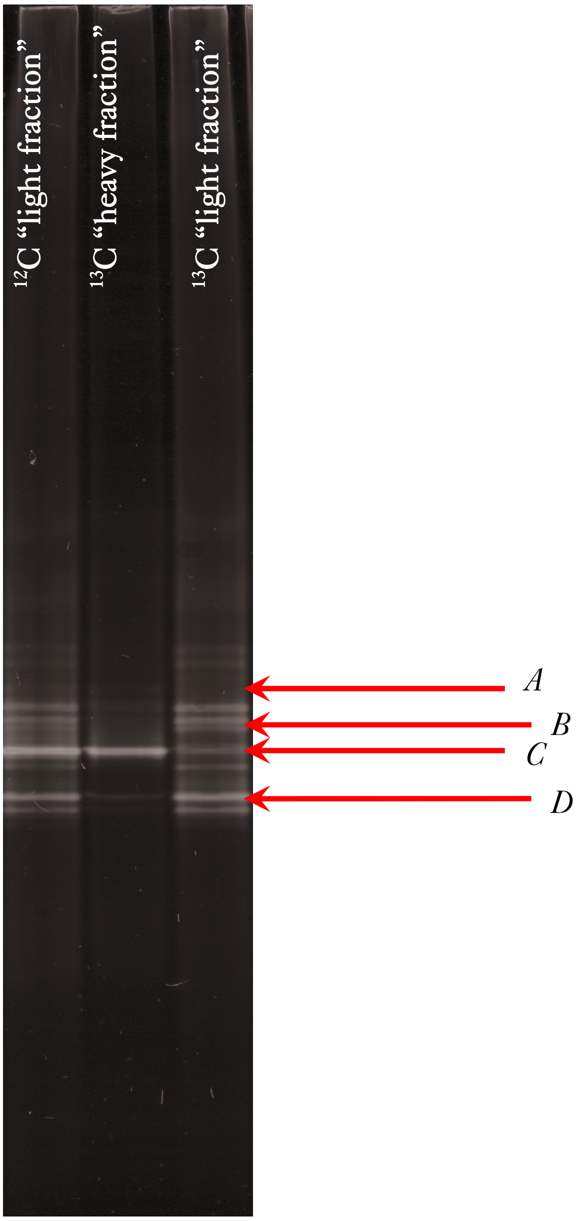

Figure 6.1. Denaturing-gradient gel electrophoretogram showing the diversity of the 16S ribosomal subunit (*rrs*) gene derived from *Bacteria* that assimilated carbon from [$^{13}C_2$]-DMS during SIP experments using water obtained from the English Channel (November 2005). See text for details of lane labels and of band identities.

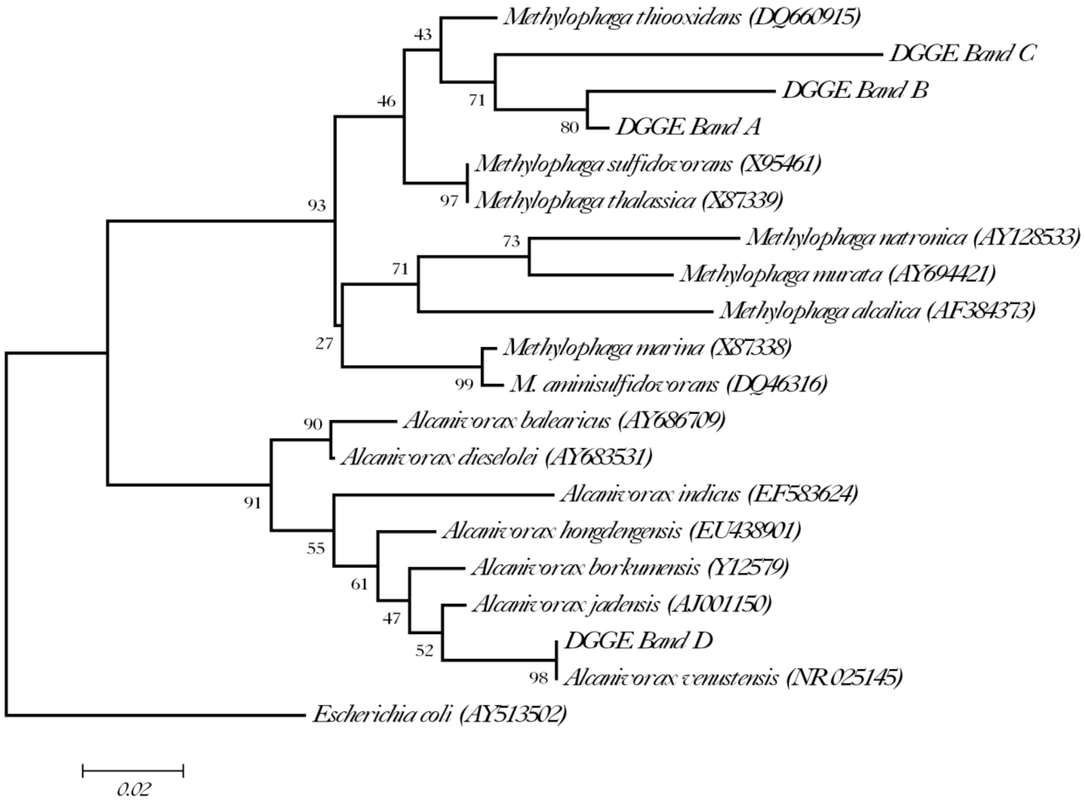

Figure 6.2. Minimum-evolution tree showing the positions of partial DNA-sequences obtained from DGGE bands to valid species of *Bacteria*. Numbers at branch points are bootstrap values from 100 replicates. Bar: 2 nucleotide substitutions per 100.

6.3 Production of tetrathionate during a phytoplankton bloom

In May 2008, artificial phytoplankton blooms were induced in 2500L aliquots of water obtained from the Raunesfjorden (Norway, 60°3'N, 5°2'E) which had undergone prefiltration (phytoplankton mesh, 0.3mm pore size) to remove larger zooplankton. A phytoplankton bloom was induced in 3 experimental mesocosms by the addition of phosphate and nitrate according to Redfield (1934). A further 3 mesocosms were left without the addition of nutrients so as to act as a control. Aliquots of water were removed from mesocosms daily and biomass was removed by passage through a PVDF filter of pore size 0.22μm. Water samples were snap-frozen in aliquots (10mL) and stored at -20°C prior to analysis. Tetrathionate and thiosulfate were quantified in bloom and control water samples obtained on each of the 12 days of the mesocosm experiment. Tetrathionate and thiosulfate concentrations over time are given in Figure 6.3.

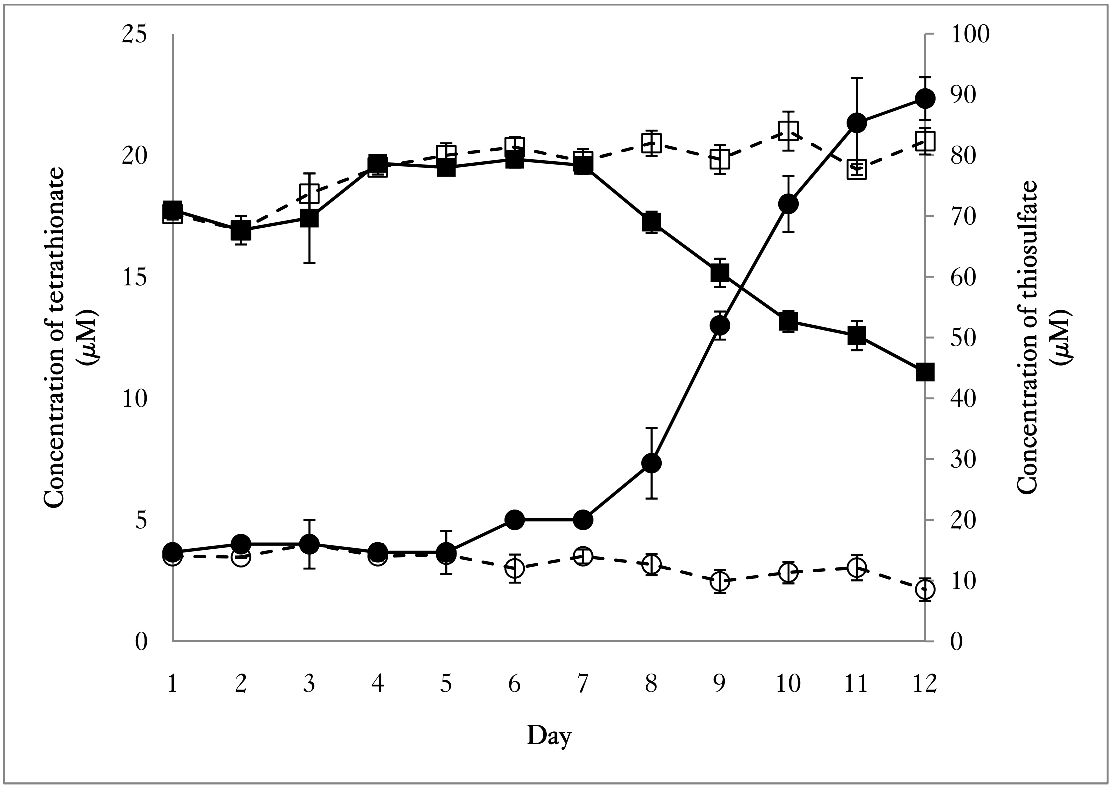

Figure 6.3. Tetrathionate and thiosulfate concentrations over the course of a 12-day induced phytoplankton bloom experiment in fjordwater in 2500L mesocosms. Filled symbols/solid lines – bloom mesocosms; hollow symbols/broken lines – control mesocosms. Circles – tetrathionate; squares – thiosulfate.

6.3.1 Tetrathionate

It can be seen from Figure 6.3 that, in mesocosms with an induced phytoplankton bloom, the tetrathionate concentration in the water column increases from Day 5 to Day 12, during which point the concentrations of DMS and DMSP in the bloom were increasing (Hatton, Mogg, Cunliffe & Murrell, *unpublished data*). In control mesocosms, tetrathionate remained relatively constant but gradually decreased over the course of the experiment. Mean tetrathionate concentrations at the beginning of the experiment were around 3.5μM, which is similar to the level of tetrathionate found in waters in the English Channel – 5.2μM in November 2008 (Boden, Kelly, Murrell & Schäfer, *unpublished data*). By the end of the experiment in bloom mesocosms, the mean concentration of tetrathionate had reached 22.3μM. Although the concentration of DMS in the water column of bloom mesocosms was in the range of 10-50nM at all times (Hatton, Mogg, Cunliffe & Murrell, *unpublished data*), the concentration of tetrathionate was much higher. If organisms capable of oxidising DMS were also able to oxidise exogenous thiosulfate to tetrathionate (as "*M. thiooxidans*" has been shown to do), it would be expected that the majority of tetrathionate produced would be from exogenous thiosulfate oxidation. Oxidation of exogenous sulfide could also provide a source of tetrathionate from these organisms. Various marine *Bacteria* such as "*Pseudomonas* 16B" (see Chapter 1) have been shown to oxidise thiosulfate to tetrathionate during heterotrophic growth. During the course of a phytoplankton bloom many compounds are released into the water column, including lipids, fatty acids, amino acids *etc.*, during the death of phytoplankton cells as well as surfactants during the growth of such cells. These compounds would be expected to act as carbon sources for heterotrophic thiosulfate oxidising *Bacteria*, resulting in a net increase in the flux of thiosulfate to tetrathionate. Thiosulfate has been previously determined in marine environments and found to be present at concentrations of up to 443μM, though it was found to be present in most samples tested at around 40-60μM (Vetter *et al.* 1989).

6.3.2 Thiosulfate

It can be seen from Figure 6.3 that, in mesocosms with an induced phytoplankton bloom, the thiosulfate concentration in the water column decreased from Day 7 to Day 12, in contrast to tetrathionate which increased. In control mesocosms, thiosulfate remained relatively constant though it was seen to accumulate from Day 1 to Day 4 in both control and experimental mesocosms. Mean thiosulfate concentrations at the beginning of the experiment were around 70μM, which is similar to the level of thiosulfate observed previously (Vetter *et al.* 1989) and found in waters in the English Channel – 62.0μM in November 2008 (Boden, Kelly, Murrell & Schäfer, *unpublished data*). By the end of the experiment in bloom mesocosms, the mean concentration of thiosulfate had decreased to 44.3μM. The drop in thiosulfate concentration by 35μM is enough to account for the formation of around 17.5μmol tetrathionate per litre, which is in good agreement with the actual increase (22.5μmol per litre).

These data suggest that, although tetrathionate is clearly produced during a phytoplankton bloom, the majority of it is formed from thiosulfate, rather than from DMS. It is known that "*M. thiooxidans*" and various marine heterotrophs (see Chapter 1) can oxidise exogenous thiosulfate to tetrathionate *via* thiosulfate dehydrogenase. The stimulation of this oxidation during the course of the bloom is likely to be simply because overall heterotroph activity would increase due to increased flux of carbon from CO_2 to DOC by the phytoplankton. Whilst tetrathionate could be being produced from DMS sulfur under these conditions, this production is "masked" by the production of tetrathionate from thiosulfate. In order to definitively show that DMS is oxidised to tetrathionate by *Bacteria* during the bloom, it would be necessary to repeat the experiment using [^{35}S]-DMS and to monitor the production of [$^{35}S_4$]-tetrathionate. It would be anticipated that some tetrathionate would be formed from both DMS-sulfur and exogenous thiosulfate, resulting in the formation of [$^{35}S_2$]-tetrathionate, in which one sulfane and the adjacent sulfonate sulfur would be labelled. At the time of writing, sodium [^{35}S]-sulfide, sodium [^{35}S]-sulfite and [^{35}S]-sulfur are not commercially available and thus the syntheses of [$^{35}S_2$]- or [$^{35}S_4$]-tetrathionate or [^{35}S]-DMS, respectively, are not possible. Additionally, the ability to oxidise thiosulfate to tetrathionate (either as a supplementary energy source or "gratuitous" oxidation) may be a more widespread trait amongst (marine) *Bacteria* than previously anticipated. Further studies into this area are necessary, particularly with respect to identifying the genes encoding thiosulfate dehydrogenase in order to allow functional microbial ecology of thiosulfate-oxidising *Bacteria*.

6.4 Summary

It has been demonstrated that *Methylophaga* spp. are dominant amongst the *Bacteria* capable of taking up [^{13}C] from [$^{13}C_2$]-DMS during SIP experiments using water obtained from the English Channel, with or without a phytoplankton bloom. Although "*M. thiooxidans*" was not found amongst 16S rRNA gene sequences in the DMS SIP experiments, this was not considered to have been likely to happen, given that "*M. thiooxidans*" was obtained from a laboratory culture some 50 years old.

Oxidation of marine thiosulfate to tetrathionate during a phytoplankton bloom was observed and found to occur at a time point correlating to the peak of the bloom at which point the populations of *Methylophaga* spp. would be expected to be at their highest. Further molecular ecology studies are underway to determine whether the increase in *Methylophaga* spp. population is indeed coupled to tetrathionate formation during the bloom.

A reassessment of heterotrophic *Bacteria* isolated from the marine environment is necessary in order to discover how widespread the thiosulfate-to-tetrathionate oxidation is in nature. The nature of this oxidation (*i.e.* gratuitous or fortuitous) needs to be assessed using chemostat culture in order to determine whether "gratuitous" sulfur-compound oxidation is a genuine physiological property or an artefact of batch-culture. Thiosulfate dehydrogenase from an heterotrophic organism needs to be purified, characterised and the genes identified. Additionally, known DMS-oxidising *Bacteria* need to be reassessed to confirm their end-product – be it sulfate, thiosulfate, tetrathionate or some other sulfur species.

CHAPTER 7

PRELIMINARY ANALYSES OF THE GENOME OF "*Methylophaga thiooxidans*"

7.1 Introduction

Since the draft genome of "*Methylophaga thiooxidans*" is available (GenBank™ accession number GG657882-GG657907), *in silico* analyses can be performed in order to obtain information on *potential* metabolic pathways, which can be used in addition to the enzyme-assay data already obtained. Text-based searches of the annotated genome were conducted to identify candidate genes, which were then searched against the GenBank™ database using the tBLASTx algorithm to verify their identity to *bona fide* genes from other species.

Various genome sequences of methylotrophic *Bacteria* are available – from the *Alphaproteobacteria* and *Gammaproteobacteria*. Additionally, some genome sequences of sulfur-oxidising *Betaproteobacteria* and *Gammaproteobacteria* are also available. Genomes of interest with respect to work on "*M. thiooxidans*" are summarised in Table 7.1.

The first draft of the "*M. thiooxidans*" genome sequence has 43 contigs of greater than 20 Sanger reads. The genome size is estimated to be greater than 3.0 Mb and has a G+C content of 46.2mol% (compared to the experimentally derived value of 45.9mol%). It can be seen from Table 7.1 that the genome of "*M. thiooxidans*" is closer in size and G+C content to sulfur-oxidising *Gammaproteobacteria* than to methylotrophic *Alphaproteobacteria* – the size difference probably being due to the heterotrophic nature of the *Methylobacterium* spp. compared to the obligately autotrophic *Thiomicrospira* sp. and *Halothiobacillus* sp. The closest related organism to "*M. thiooxidans*" with an available genome sequence for comparison is

Thiomicrospira crunogena, which has a G+C content of 43mol% (compared to 46.2mol% for "*M. thiooxidans*") and a genome size of 2.4Mb (compared to around 3.0Mb).

Organism	Length (Mb)	G+C content (mol%)	% Coding	No. of genes	No. of protein-coding genes	No. of structural RNAs	No. of pseudogenes	No. of other genes
Methylobacterium chloromethanicum	5.7	68	83	5464	5173	79	212	7
Methylobacterium populi	5.8	69	85	5492	5314	76	102	11
Methylobacterium sp. 4-46	7.7	71	81	7047	6609	83	355	11
Methylobacterium radiotolerans	6.0	71	86	5839	5686	71	82	7
Methylobacterium extorquens PA1	5.5	68	83	4956	4829	75	52	7
Methylobacterium nodulans	7.7	68	81	7767	7355	94	318	13
Methylocella sylvestris BL2	4.3	63	85	3977	3818	57	102	5
Thiobacillus denitrificans	2.9	66	92	2879	2827	52	0	0
Halothiobacillus neapolitanus	2.5	54	89	2481	2438	43	0	0
Thiomicrospira crunogena XCL-2	2.4	43	89	2259	2196	55	8	4

Table 7.1. Properties of genome sequences from methylotrophic *Alphaproteobacteria* (*Methylobacterium* spp.) and sulfur oxidising *Betaproteobacteria* and *Gammaproteobacteria* (*Thiobacillus* sp., *Halothiobacillus* sp. and *Thiomicrospira* sp.).

In terms of pathways of interest that were searched for in the genome, the pathways of C_1 metabolism (*i.e.* methanol oxidation, methylated amine oxidation, formaldehyde oxidation and the KDPG RuMP pathway) were prioritised. Genes encoding key proteins of nitrogen and sulfur metabolism were selected due to central metabolic interest. Genes encoding Krebs' cycle and the metabolism of sugars (since the organism grows on fructose and other polycarbon compounds) were also investigated. Due to the speculation that "*M. thiooxidans*" may use a corrinoid-dependent demethylase during DMS metabolism, cobalt uptake pathways were also investigated.

7.2 Methanol oxidation pathways

The *mxaF*, *mxaI* and *mxaR* genes from the cluster *mxaFJGIRSACKLDEK* present in methylotrophs and methanotrophs (Amaratunga *et al.* 2006) have been identified in the draft genome of "*M. thiooxidans*"; however, the rest of the cluster is absent in the incomplete genome. The genes *mxaFI* encode the methanol dehydrogenase enzyme, the activity of which has been confirmed by enzyme assay in methanol and DMS-grown cells (Chapter 5). In terms of the nearest validated species neighbour, MxaF from "*M. thiooxidans*" shows 69% identity to that of *Methylocella sylvestris* (CP001280). Additionally, the *xoxFI* cluster previously identified as being expressed in "*M. thiooxidans*" during growth on DMS and methanol (Schäfer 2007) has been identified. Genes encoding enzymes for the biosynthesis of PQQ (co-factor of methanol dehydrogenase) have been identified in a cluster *pqqBCDE*; however, the *pqqA* gene appears to be absent. Though this could be due to the incomplete nature of the genome at the moment, *pqqA* was also noted to be absent in the genome of *Methylocella silvestris* BL2 (Chen 2008). In *Klebsiella pneumoniae*, the PQQ biosynthesis genes are clustered as *pqqABCDEF* (Meulenberg *et al.* 1992), whereas in *Pseudomonas aeruginosa*, the *pqqABCDE* operon is separated from the *pqqF* gene (Gliese *et al.* 2004). In *M. extorquens*, the *pqqABC/DE* operen (with *pqqC* and *pqqD* fused into a single protein) is separated from the *pqqFG* genes, which are located in a separate operon with other genes (Zhang & Lidstrom 2003). In all organisms characterised to date, with the exception of *Methylocella silvestris*, the *pqqA* gene is present and has recently been shown to be essential for PQQ biosynthesis, though the role of the PqqA *in vivo* remains unknown (Hölscher & Görisch 2006). In terms of the nearest validated species neighbour, the PqqB polypeptide from "*M. thiooxidans*" shows 69% identity to that of *Methylococcus capsulatus* (Bath) (AE017282).

7.3 Methylated amine oxidation pathways

The *mauBEDAMN* operon encoding the monomethylamine dehydrogenase and associated proteins used in the metabolism of MMA, DMA and TMA (van der Palen *et al.* 1995) has been identified in the genome. Although "*M. thiooxidans*" can use all three methylated amines as sole carbon and nitrogen sources, the genes encoding dimethylamine dehydrogenase (*dmd*) (Yang *et al.* 1995) and trimethylamine dehydrogenase (*tmd*) (Boyd *et al.* 1992) do not appear to be present in the incomplete genome. The MauB polypeptide shows 70% identity to that of *Methylophilus methylotrophus* (L26407).

7.4 C_1 metabolism and the RuMP pathway

Some of the genes encoding formate dehydrogenase (*fahEAB*) and formaldehyde-activating enzyme (*faeA*) are present in the genome.

It has been shown by enzyme-assay that the KDPG-aldolase variant RuMP pathway of formaldehyde assimilation is likely to be used by "*M. thiooxidans*". Of the enzymes of the RuMP pathway, three were absent from the draft genome – transaldolase, 3-hexulose-6-phosphate synthase and glucose-6-phosphate dehydrogenase. KDPG aldolase was present but fructose-1,6-bisphosphate aldolase was absent – consistent with enzyme assay data and the physiology of other *Methylophaga* spp (Chapter 5; Janvier & Grimont 1995).

7.5 Nitrogen metabolism

"*M. thiooxidans*" can use urea, nitrate and ammonium as sole nitrogen sources during growth (Chapter 5).

Ammonium transporters have been identified in the genome, along with *glnB* which encodes a putative nitrogen regulatory protein linked to ammonium sensing (Kennedy *et al.* 1994). A cluster of genes *glnTADBST* encoding genes of the glutamine synthase pathway of nitrogen assimilation has been identified, however, the *glnK* regulatory gene appears to be absent.

The urease-encoding gene, *ureB* (Labigne *et al.* 1991), was found to be present, indicating the ability of "*M. thiooxidans*" to hydrolyse urea to ammonia and carbon dioxide in the first step of the pathway of nitrogen assimilation from urea. Urease activity has been confirmed in the organism by the routine urease test using MAMS agar (made without ammonium chloride) containing phenol red and supplemented with fructose and urea as the sole nitrogen source. The rapid increase in pH (*i.e.* turning the agar magenta) during growth was taken to be indicative for ammonia formation from urea.

Interestingly, one of the genes encoding cyanate hydratase, *cynS* (Guilloton *et al.* 1993), has been identified in the genome; however, "*M. thiooxidans*" could not use cyanate as a nitrogen source during growth on methanol.

Nitrate reductase (*narHGI*) has been identified in the genome sequence, in addition to *nirK*, the dissimilatory nitrate reductase involved in denitrification - *Methylophaga* spp. are known to denitrify as far as nitrite (Chapter 5).

7.6 Sulfur metabolism

Of the genes encoding the Kelly-Friedrich pathway of thiosulfate oxidation, *soxXYZABCDEF* (Friedrich *et al.* 2000), only the gene encoding the sulfur chelating protein *soxZ* appears to be present. Although the Kelly-Friedrich pathway is not involved in sulfur oxidation in "*M. thiooxidans*" (Chapter 5), the genes encoding it are known to be widespread in various groups of *Bacteria* – even in ones that apparently do not oxidise reduced sulfur compounds (Friedrich *et al.* 2001). The SoxZ polypeptide from "*M. thiooxidans*" shows 67% identity to that of *Methylococcus capsulatus* Bath (AE017282).

A gene encoding rhodanese, *rhdA* (Colnaghi *et al.* 1994) has been found in the genome; however, it does not seem to be expressed during growth of "*M. thiooxidans*" on methanol or DMS as the enzyme activity is absent. Assuming the *rhdA* gene in "*M. thiooxidans*" encodes an active rhodanese enzyme, it is likely that it is involved in cyanide detoxification or in lipoate metabolism.

A gene encoding sulfate permease (*cysA*), which is involved in the uptake of sulfate and thiosulfate, was found in the genome, consistent with the use of sulfate as a sole sulfur source during growth and the use of exogenous thiosulfate as a supplementary energy source during growth on methanol *etc*. Genes encoding PAPS reductase (*cysH*), APS kinase (*cysC*) and sulfite reductase (*cysJ*) from the sulfate assimilation pathway were also found (Schmidt & Jäger 1992) in the genome.

The one known gene encoding a key step in the DMS oxidation pathway (sulfide dehydrogenase, *sud*) was absent. Two ORFs were found which were similar to the methanethiol oxidase gene, *mto*, from *Hyphomicrobium* VS (Myronova, Boden & Schäfer, *unpublished data*).

7.7 Krebs' Cycle

Since "*M. thiooxidans*" can use multi-carbon compounds as a carbon source (*e.g.* fructose, substituted thiophenes), it would be anticipated that the Krebs' cycle of carbohydrate metabolism (Krebs & Johnson 1937) would be functional in this organism. Of the enzymes of the Krebs' cycle, all but two were present in the draft genome – succinate thiokinase and malate dehydrogenase. The glyoxylate shunt across the cycle used in acetate metabolism is comprised of the enzymes isocitrate lyase and malate synthase. Both of these enzymes are absent from the draft genome, which is not surprising since the organism does not grow on acetate.

7.8 Glycolysis

Growth on sugars by "*M. thiooxidans*" seems to be restricted to fructose – out of 9 sugars assessed. Of the two major glycolytic pathways found in the *Bacteria* - the Embden-Meyerhof-Parnas (Embden & Oppenheimer 1912) and Entner-Doudoroff (Entner & Doudoroff 1952) pathways - the former is absent in many methylotrophic organisms owing to the absence of fructose-1,6-bisphosphate (FBP) aldolase (van Dijken & Quayle 1977, Green & Gibson 1984).

The majority of the genes encoding enzymes of the Entner-Doudoroff pathway were found in the draft genome, with the exception of gluconate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase.

Over half of the genes encoding the enzymes of the Embden-Meyerhof-Parnas pathway were found in the draft genome – those not found were hexokinase, phosphohexose isomerise, glucose-6-phosphatase, phosphofructokinase and fructose-1,6-bisphosphate aldolase. Given that *Methylophaga* spp. use the KDPG-variant RuMP pathway rather than the FBP aldolase variant (Janvier & Grimont 1995), it is likely that FBP aldolase is absent from the organism, rather than merely being absent from the draft genome.

7.9 Cytochromes

21 putative cytochrome encoding genes were found in the draft genome. In addition to the bc_1 complex, a total of 12 cyt *c* (mainly c_{553}) and 7 cyt *b* (mainly b_{561}) were found, along with bacterioferritin.

7.10 Cobalt metabolism

Given that DMS demethylase is likely to be corrinoid-dependent, enzymes of cobalt metabolism are of interest. A syrohydrochlorin cobaltochelase (*cbiX*) involved in cobalt uptake was found, in addition to *cob* genes involved in the biosynthesis of corrinoids (Raux *et al.* 1998), including the cyanide-eliminating cyanocobalamin reductase involved in the conversion of cyanocobalamin to functional vitamin B_{12}. This may explain the presence of the gene encoding rhodanese – to detoxify the cyanide ions formed during the reduction of cyanocobalamin.

7.11 Conclusions and perspectives

The genome sequences of "*M. thiooxidans*" DMS010^T will be completed and closed in the next few years. Then, searches for the apparently "missing" genes of the major metabolic pathways need to be performed. In any case, the presence or absence of any of the enzymes identified in terms of their encoding genes in the genome needs to be confirmed in terms of enzyme activity.

As identified elsewhere (Chapter 5), purification of the various enzymes of DMS oxidation in this species need to be attempted in order to identify the genes encoding them. Once identified, the regions up- and downstream of these genes need to be studied in order to understand their regulation and accessory proteins (such as cytochromes).

Although attempts have been made to study comparative proteomics of "*M. thiooxidans*" grown on a variety of substrates (Boden, Patel, Slade & Schäfer, *unpublished data*), the incomplete nature of the genome has limited this work.

After DMS, the main priorities with respect to pathways which can be better understood from proteomics/genomics of this organism are the mechanisms of thiophene metabolism. The range of organisms isolated which can grow on substituted thiophenes as a carbon source is currently limited to *Flavobacterium* sp. (Amphlett & Callely 1969), organism R1 (Cripps 1973), *Rhodococcus* sp. TTD-1 (Kanagawa & Kelly 1987), *Xanthobacter tagetidis* (Padden *et al.* 1997), *Sphingomonas melonis* ET35 (Boden *et al.* 2008) and "*M. thiooxidans*". Of these organisms, "*M. thiooxidans*" is the only one with a genome sequence available. Thiophenes are an important class of plant secondary metabolite and represent a virtually uncharacterised transfer of sulfur from plants to the soil. Thiophenes are based on a five-membered thiophene ring and can take a variety of substitutions on both the carbon and sulfur atoms.

Thiophenes can be oxidised to 2-oxoglutarate, which is fed into Krebs' cycle and assimilated to biomass (Cripps 1973). The sulfur is ultimately oxidised to sulfate, in *X. tagetidis* and organism R1 (Padden *et al.* 1997; Cripps 1973) though the mechanisms of this remain unclear. "*M. thiooxidans*" is an ideal organism in which to elucidate the pathways of thiophene metabolism owing to its rapid growth-rate on thiophene-3-carboxylate ($\mu_{max} > 0.30h^{-1}$) and the availability of the genome sequence. Once the genome sequence has been completed, further studies (for example, by proteomics or transcriptomics) may reveal the genes responsible for thiophene metabolism, the pathways of which were first postulated 40 years ago (Cripps 1973) and on which virtually no progress has been made.

CHAPTER 8

DIMETHYLSULFIDE AS AN ENERGY SOURCE DURING THE CHEMOORGANOHETEROTROPHIC GROWTH OF *Sagittula stellata* E-37

8.1 Introduction

Sagittula stellata (Figure 8.1) is a heterotrophic marine member of the *Alphaproteobacteria* which has been shown to oxidise DMS to DMSO during heterotrophic growth on glucose but is unable to grow on DMS as a sole source of carbon and energy (González *et al.* 1997; González *et al.* 1999). The mechanism of and purpose for DMS oxidation in *S. stellata* remains uncharacterised. The genome sequence of *S. stellata* E-37^T is available (AAYA00000000) and is approximately 5.3Mb in size, with 5067 predicted protein coding genes, out of a total of 5112 genes.

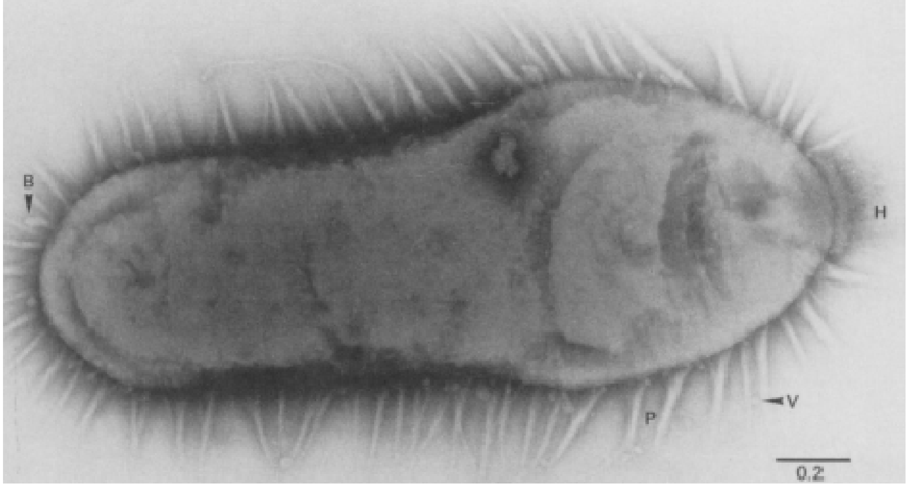

Figure 8.1 Ultrastructure of *Sagittula stellata* E-37^T. Scale bar represents 0.2μm. (from González *et al.* 1997, Copyright © 1997 International Union of Microbiological Societies).

DMS dehydrogenase (DdhABC) is an heterotrimeric molybdopterin-dependent enzyme which has been purified from *Rhodovulum sulfidophilum* (McDevitt *et al.* 2002) and has been shown to catalyse the reaction:

$$(CH_3)_2S + O_2 + 2H^+ \rightarrow (CH_3)_2SO + H_2O$$

DMS dehydrogenase is well-characterised and has been shown to be encoded by the operon *ddhABDC* (McDevitt *et al.* 2002). *ddhA*, *ddhB* and *ddhC* encode the three (alpha-, beta- and gamma-) subunits of the enzyme whereas *ddhD* encodes a protein thought to be involved in DdhABC assembly *in vivo*.

Searching for *R. sulfidophilum* DdhABC amino acid sequences against the translated *S. stellata* genome using the BLASTp algorithm revealed predicted proteins with >55% identity to DdhABC, clustered together and annotated as components of a nitrate reductase (NarYZV, EBA07058-EBA07060). It is worth noting that *S. stellata* apparently does not reduce nitrate (González *et al.* 1997) during heterotrophic growth.

The physiological role of DdhABC in *R. sulfidophilum* is to catalyse the oxidation of DMS to DMSO during photoorganoheterotrophic growth. Electrons liberated during the oxidation of DMS to DMSO are passed into the light-driven electron transfer cycle at the level of cytochrome c_2 (McDevitt *et al.* 2002). No cytochrome encoding genes appear to be located in the proximity of the *narYZV* cluster in the *S. stellata* genome.

A possible role of DMS oxidation in *S. stellata* is to provide additional energy during chemoorganoheterotrophic growth. If this were the case, it would be probable that a DMS dehydrogenase enzyme, possibly encoded by the *narYZV* genes, was responsible for the oxidation of DMS to DMSO.

Additionally, genes annotated as a DMSO reductase-like molybdopterin-containing dehydrogenase are also present in the genome (EBA06368 - EBA06370); a tBLASTx search against the GenBank™ database confirms the annotation.

8.2 Growth in chemostat culture

To assess the effects of DMS on the growth of *S. stellata*, experiments were conducted in fructose- and succinate- limited chemostats with or without the addition of DMS.

8.2.1 Growth under fructose limitation

Steady-state cultures were established with 12mM fructose as the growth-limiting substrate at dilution rates (*D*) between $0.01h^{-1}$ and $0.15h^{-1}$, between which the steady-state growth yield (*Y*) increased from 3.8 to 29.7g dry biomass per mole of fructose. The maximum growth yield (Y_{max}) was estimated from a plot of $1/Y$ *versus* $1/D$ as 64.2g dry biomass per mole of fructose (10.7g dry biomass per mole of substrate carbon). From washout kinetics, μ_{max} was found to be $0.17h^{-1}$. From a plot of q *versus* D, m_S was found to be 2.5mmol fructose $g^{-1}\cdot h$.

From the genome sequence of *S. stellata*, all of the genes encoding the enzymes of both the Entner-Doudoroff and Embden-Meyerhof-Parnas pathways of glycolysis appear to be present in the organism. The theoretical Y_{max} for fructose dissimilated to PGA *via* the Entner-Doudoroff pathway is 73.7g dry biomass per mole of fructose (12.3g dry biomass per mole of substrate carbon) whereas that for fructose dissimilation to PGA *via* the Embden-Meyerhof-Parnas pathway is 122.4g dry biomass per mole of fructose. The Y_{max} observed during the growth of *S. stellata* under fructose-limitation is closer to that predicted for dissimilation *via* the Entner-Doudoroff pathway than the Embden-Meyerhof-Parnas, suggesting that the former is probably the pathway in use in this case. It should be noted, however, that many *Bacteria* use multiple pathways of glycolysis simultaneously during growth on hexoses (Wood *et al.* 1977).

8.2.2 Addition of DMS to a fructose-limited chemostat

The effects of DMS on *S. stellata* grown under fructose-limitation were assessed. A steady-state was established on fructose (D = 0.03h^{-1}, S_0 = 12mM) and Y determined before DMS was added to the vessel to a final concentration of 1mM and 5 culture volume changes were allowed before redetermination of Y along with concentrations of fructose, DMS, DMSO and DMSO$_2$. Upon addition of DMS, there was a delay in the perturbation of the steady-state, with no decrease in dissolved-oxygen concentration of the culture observed until approximately 6h after the beginning of the addition of DMS. At steady-state DMS and DMSO$_2$ were not detected in the culture, though DMSO was present at 0.9mM (± 0.03mM, n = 3). Y was found to increase from 10.5 to 12.2g dry biomass per mole fructose (1.8 to 2.0g dry biomass per mole of fructose carbon).

In order to assess the effects of DMS on Y_{max} and m_S, steady-states were established between D = 0.01h^{-1} and 0.15h^{-1} on fructose (S_0 = 12mM) with and without the addition of 1mM DMS. At each steady-state, Y was determined and the concentrations of DMS and DMSO measured. The increase in $Y_{\max}$ per mole of DMS oxidised was from 64.2 to 73.2g dry biomass per mole of fructose (10.7 to 12.2g dry biomass per mole of substrate carbon). At all dilution rates, DMS was found to be completely oxidised to DMSO at steady-state. Whilst $Y_{\max}$ increased, it was found that m_S remained constant at 2.5mmol fructose $g^{-1} \cdot h$, as was found to be the case when thiosulfate was used as an auxiliary energy source by "*Methylophaga thiooxidans*" (Chapter 5). The $Y_{\max}$ in the presence of DMS was closer to the theoretical $Y_{\max}$ than that observed in the absence of DMS, indicating a tighter coupling of fructose oxidation to growth in the presence of DMS, with less dissimilation of PGA to carbon dioxide to meet the energy requirements of growth and maintenance.

8.2.3 Growth under succinate-limitation

Steady-state cultures were established with 2mM succinate as the growth-limiting substrate at D between $0.01h^{-1}$ and $0.10h^{-1}$, between which Y increased from 2.9 to 21.0g dry biomass per mole of succinate. The maximum growth yield ($Y_{\max}$) was estimated from a plot of $1/Y$ *versus* $1/D$ as 33.6g dry biomass per mole of succinate (8.4g dry biomass per mole of substrate carbon). From washout kinetics, $\mu_{\max}$ was found to be $0.12h^{-1}$. From a plot of q *versus* D, m_S was found to be 3.2mmol succinate $g^{-1} \cdot h$.

The theoretical Y_{max} for succinate (assuming 32% of succinate carbon is assimilated to biomass, as calculated by Anthony (1982) for a range of organisms) is 37.1g dry biomass per mole of succinate (9.23g dry biomass per mole of substrate carbon).

8.2.4 Addition of DMS to a succinate-limited chemostat

The effects of DMS on *S. stellata* grown under succinate-limitation were assessed. A steady-state was established on fructose ($D = 0.03h^{-1}$, $S_0 = 2mM$) and Y determined before DMS was added to the vessel to a final concentration of 1mM and 5 culture volume changes were allowed before redetermination of Y along with concentrations of succinate, DMS, DMSO and $DMSO_2$. Upon addition of DMS, there was, as previously observed upon the addition of DMS to a fructose-limited chemostat, a delay in the perturbation of the steady-state, with no decrease in dissolved-oxygen concentration of the culture observed until approximately 3h after the beginning of the addition of DMS – approximately half of the time taken for the perturbation of a fructose-limited steady-state to occur. At steady-state DMS and $DMSO_2$ were not detected in the culture, though DMSO was present at 0.9mM (± 0.02mM, $n = 3$). Y was found to increase from 7.3 to 8.2g dry biomass per mole succinate (1.8 to 2.0g dry biomass per mole of succinate carbon).

In order to assess the effects of DMS on Y_{max} and m_S, steady-states were established between $D = 0.01h^{-1}$ and $0.10h^{-1}$ on succinate ($S_0 = 2mM$) with and without the addition of 1mM DMS. At each steady-state, Y was determined and the concentrations of DMS and DMSO measured. The increase in Y_{max} per mole of DMS oxidised was from 33.6 to 38.9g dry biomass per mole of succinate (8.4 to

9.7g dry biomass per mole of substrate carbon). At all dilution rates, DMS was found to be completely oxidised to DMSO at steady-state. Whilst Y_{max} increased, it was found that m_{S} remained constant at 3.2mmol succinate $\mathrm{g}^{-1}\cdot\mathrm{h}$, as was found to be the case when the organism was grown under fructose-limitation in the presence of DMS. As with a culture grown under fructose-limitation, the Y_{max} in the presence of DMS was closer to the theoretical Y_{max} than that observed in the absence of DMS, again indicating a tighter coupling of substrate oxidation to growth in the presence of DMS.

8.3 Enzymes of DMS metabolism

Since genes similar to those encoding DMS dehydrogenase and DMSO reductase have been identified in the genome of *S. stellata*, it was felt likely that one or both of these enzymes was responsible for DMS oxidation to DMSO (with DMSO reductase working in reverse). Enzyme assays were conducted using CFEs prepared from cells obtained from a succinate-limited chemostat and activities are given in Table 8.1.

Organism	Specific activity [nmol substrate oxidised min^{-1}/(mg protein)$^{-1}$]	
	DMS dehydrogenase (DCPIP)	DMSO reductase (MV)
Sagittula stellata	1 (±1)	0 (±0)
Rhodovulum sulfidophilum	24 (±4)	0 (±0)
Hyphomicrobium sulfonivorans	0 (±0)	57 (±3)

Table 8.1 Specific activities of enzymes of DMS oxidation in CFEs prepared from cells of *Sagitulla stellata* E-37 obtained from a succinate-limited chemostat (D = 0.03h^{-1}, S_0 = 2mM) with DMS as an auxiliary substrate. Positive controls are *Rhodovulum sulfidophilum* grown photoorganoautotrophically with DMS as an electron donor (DMS dehydrogenase) and *Hyphomicrobium sulfonivorans* grown heterotrophically on $DMSO_2$ (DMSO reductase). Figures in brackets are standard error of mean (n = 3).

It can be seen from Table 8.1 that DMS dehydrogenase and DMSO reductase activities are both absent from *S. stellata* grown on succinate in the presence of DMS as an auxiliary energy source. It is entirely possible that a DMS dehydrogenase is present and active in *S. stellata* grown under these conditions *in vivo*, but that it does not couple to DCPIP *in vitro*, or that the enzyme is highly unstable and that activity is lost during the preparation of CFEs. Since the DMS dehydrogenase in *R. sulfidophilum* is coupled to cytochrome c_2 *in vivo*, ferricyanide was assessed in place of DCPIP as an *in vitro* electron acceptor; however, no activity was observed in *S. stellata* CFE, though in *R. sulfidophilum* CFE, an activity of 36 (±3) nmol ferricyanide reduced min^{-1} (mg protein)$^{-1}$ was observed.

In order to investigate if this was the case, protein profiling of *S. stellata* grown on succinate with and without the presence of DMS was performed.

8.4 Protein profiling of *S. stellata* grown in the presence and absence of DMS

CFEs were prepared from cells of *S. stellata* obtained from succinate-limited chemostats (D = 0.03h^{-1}, S_0 = 2mM) with and without DMS (1mM) as an auxiliary energy source. CFE proteins were denatured using lithium dodecyl sulfate and dithiothreitol before separating using NuPAGE™. A NuPAGE™ gel showing separated proteins is shown in Figure 8.2.

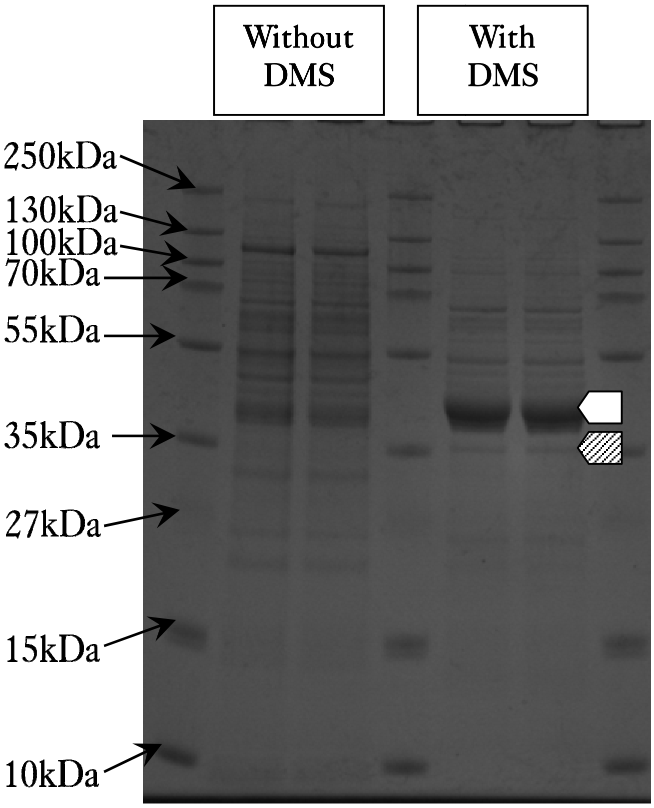

Figure 8.2 Silver-stained NuPAGE™ gel of CFEs prepared from cells of *S. stellata* obtained from succinate-limited chemostats (D = 0.03h^{-1}, S_0 = 2mM) grown with and without the presence of DMS as an auxiliary energy source (pairs of lanes are duplicates of the same CFE sample). The white and hashed arrowheads refer to polypeptides "X" and "Y", respectively, as discussed in the text.

It can be seen from Figure 8.2 that two polypeptides are expressed at higher levels in the presence of DMS – X and Y as indicated by the shaded arrowheads.

Polypeptide X is approximately 42kDa and Polypeptide Y is approximately 35kDa. Bands were excised from the gel, destained with ferricyanide, digested with bovine pancreatic trypsin and subjected to LC-ESI-MS/MS analysis, with data searched against proteins predicted from the *S. stellata* genome sequence and the UniProt™ database. Whilst peptides derived from Polypeptide X showed identity (91%) to malate dehydrogenase from *S. stellata* (EBA08124), those from Polypeptide Y showed no significant matches. This is likely to be due to poor elution of the digested peptides from the acrylamide gel matrix (Susan E Slade, *personal communication*), usually due to the digested fragments being particularly hydrophobic.

8.5 Malate dehydrogenase

A conserved-domain search (Marchler-Bauer *et al.* 2007) of the EBA08124 polypeptide reveals that it is a member of the lactate dehydrogenase-like malate dehydrogenases. These dehydrogenases show structural similarity to lactate dehydrogenases but have no lactate dehydrogenase activity – instead having malate dehydrogenase activity, catalysing the reaction:

$$\text{Malate} + \text{NAD}^+ \rightarrow \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

Malate dehydrogenase activity was measured in CFEs prepared from cells obtained from a succinate-limited chemostat without or with DMS and was found to be 112 ± 4 nmol NADH formed min^{-1} (mg protein)$^{-1}$ or 672 ± 4 nmol NADH formed min^{-1} (mg protein)$^{-1}$, respectively ($n = 3$). In order to ascertain whether

DMS can act as an alternative substrate for malate dehydrogenase, 350μM DMSO was used in place of 350μM oxaloacetate in the assay (Borodina *et al.* 2000). No activity was recorded with DMSO in CFEs corresponding to either growth condition. A further assay was conducted using NAD$^+$ in place of NADH and DMS in place of malate to monitor NAD$^+$-dependent DMS dehydrogenase activity. No activity was recorded in CFEs corresponding to either growth condition.

8.6 Coupling of DMS oxidation to ATP production

ATP-production experiments were conducted in identical conditions to those outlined in Chapter 5 using *S. stellata* cells obtained from a succinate-limited chemostat grown in the presence of DMS. DMS (1mM) was added to each aliquot of cells in place of thiosulfate and ATP formation monitored over time. DMS-dependent formation of ATP is shown in Figure 8.3.

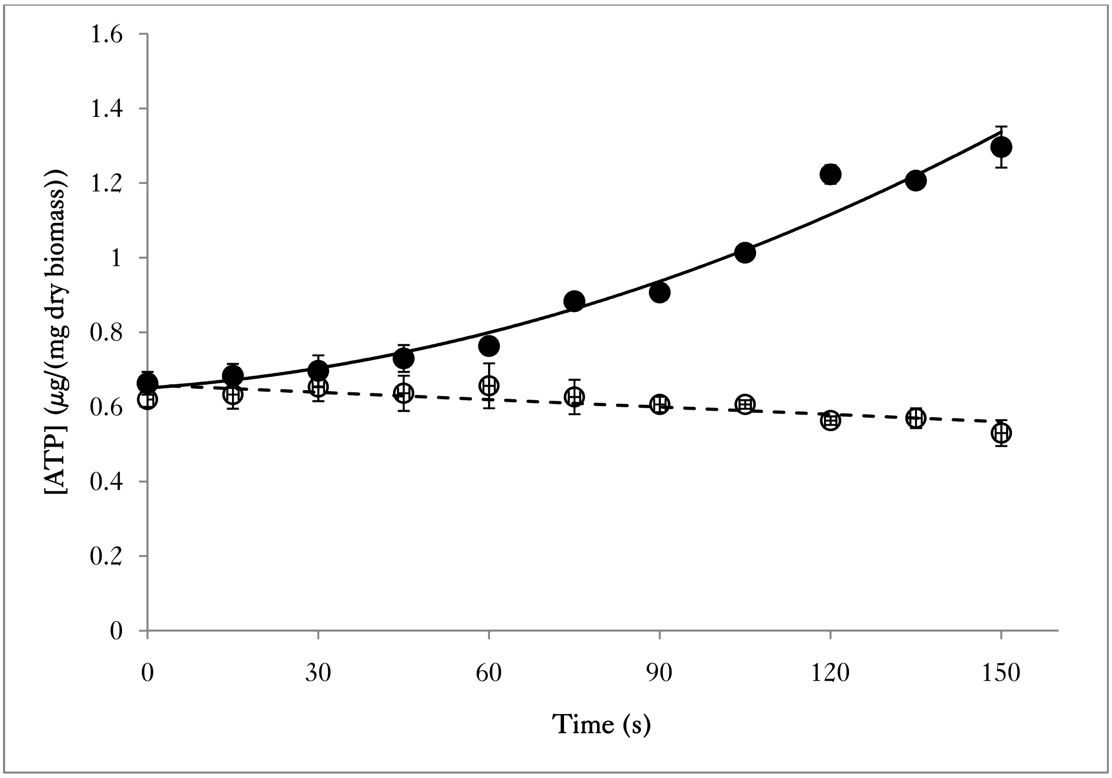

Figure 8.3 ATP production by cells of *S. stellata* obtained from a succinate-limited chemostat grown in the presence of 1mM DMS. Hollow circles/broken line represent control incubations without exposure to DMS; solid circles/solid line represent experimental incubations with 1mM DMS. Error bars indicate standard error of mean ($n = 7$).

It can be seen from Figure 8.3 that ATP is produced in the presence of DMS by cells of *S. stellata* – approximately 0.20mg ATP (mg dry biomass)$^{-1}$ being formed per mole of DMS oxidised to DMSO. It is interesting to note that the production of ATP here apparently follows an exponential rather than logarithmic pattern (as observed in "*Methylophaga thiooxidans*" and *Halothiobacillus neapolitanus* during thiosulfate oxidation – *cf.* Chapter 5). There is also a lag-phase before ATP production begins, suggesting that the oxidation of DMS is not immediate and that DMS must first be transported into the cells – possibly by active transport. This is in contrast to the immediate thiosulfate oxidation in "*M. thiooxidans*" and *H. neapolitanus*, which is thought to occur in the periplasm.

The amount of ATP produced per mole of energy source here is 0.20mg (mg dry biomass)$^{-1}$ – which is in the same order of magnitude as that produced from thiosulfate oxidation by "*M. thiooxidans*" (0.13mg) or *H. neapolitanus* (0.26mg).

8.7 Discussion

The oxidation of DMS to DMSO by *Sagittula stellata* has been demonstrated to be linked to an increase in Y_{max} – indicative of chemoorganoheterotrophic growth. The increase in Y_{max} is around 15% in cells grown under both fructose-limitation and succinate-limitation. ATP synthesis was found to be coupled to DMS oxidation – a schematic showing the potential pathway for electron transfer is given in Figure 8.4. The enzyme responsible for the oxidation of DMS to DMSO remains unknown at this stage; however, it is possible that a molybdopterin-containing dehydrogenase similar to DMS dehydrogenase could be responsible but is not being detected under the assay conditions due to the requirement of an alternative *in vitro* electron acceptor (*e.g.* Wurster's blue, brilliant cetyl blue, methylene blue, Lauth's violet, benzoquinone) for activity. Alternatively, the DMS dehydrogenase in *S. stellata* could be particularly unstable and damaged during the process of preparing CFEs.

It is not known if the electron transfer from the DMS oxidising enzyme is directly onto the cytochromes of the respiratory chain or *via* the quinone pool, though this could be investigated by way of cell spectra being taken in the presence or absence of myxothiazol or antimycin A, which inhibit transfer *via* the quinone pool.

Attempts were made at taking cell spectra; however, those taken in the presence of DMS were subject to noise and it was not possible to obtain high-quality spectra at 77K due to difficulties in preparing clear ethylene glycol glasses from extracts. Further optimisation of the glassing of *S. stellata* extracts is required in order to obtain meaningful spectra.

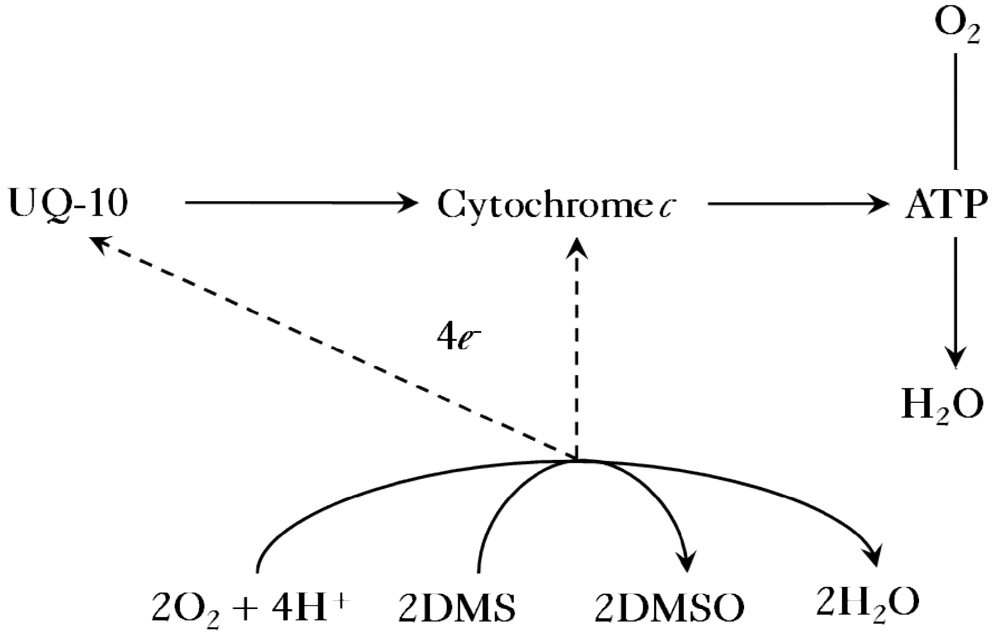

Figure 8.4. A schematic representation of the potential pathway of DMS oxidation in *Sagittula stellata* E-37 and its coupling to ATP synthesis, balanced with respect to respiratory oxygen. Broken lines represent potential electron transfer steps from the DMS-oxidising enzyme to the quinone pool or cytochromes of the respiratory chain.

Malate dehydrogenase (EC. 1.1.1.37) was found to be expressed at higher levels during growth on succinate in the presence of DMS *versus* growth without DMS in terms of both the increased amount of the protein observed by NuPAGE™ analysis and in terms of enzyme activity. No NAD^+-linked DMS dehydrogenase activity (*i.e.* potentially catalysed by malate dehydrogenase) was observed in *S. stellata* grown in the presence of DMS – either in terms of DMS-dependent NAD^+ reduction or DMSO-dependent NADH-oxidation.

Whilst malate dehydrogenase is upregulated in the presence of DMS, it appears to have no role in DMS oxidation. Assuming that succinate-grown cells assessed were using Krebs' cycle for succinate metabolism, malate dehydrogenase is the next step that generates reducing equivalents after succinate enters the cycle and occurs before a limiting step (citrate synthase), in which oxaloacetate would build up if the specific activity of malate dehydrogenase were higher than that of citrate synthase resulting in oxaloacetate being diverted into other pathways (*e.g.* into aspartate biosynthesis) or building up within cells. Of the three steps in Krebs' cycle in which reducing equivalents are formed (isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase and malate dehydrogenase), malate dehydrogenase is the only one in which reducing equivalents are generated without the liberation of carbon dioxide. If malate dehydrogenase is upregulated during oxidation of DMS, more reducing equivalents would be anticipated to be generated by this enzyme, probably reducing the amount of carbon dioxide liberated from Krebs' cycle, thus allowing biosynthesis from isocitrate and 2-oxoglutarate (*e.g.* glutamine synthesis), rather than oxidation to carbon dioxide and reducing equivalents. This conservation of carbon and enhanced production of reducing equivalents may account for some of the increase in Y_{max} observed in the presence of DMS. The regulation of this process remains unknown and it is not currently clear whether DMS itself or its oxidation to DMSO regulates the increase in malate dehydrogenase activity.

Rather than the oxidation of DMS to DMSO providing electrons to the respiratory chain for ATP synthesis, it is possible that a more complex system of energy gain is in use by *S. stellata*. The oxidation of DMS to DMSO alone only provides 2mols of electrons per mole of DMS oxidised – perhaps not sufficient to provide a 15% increase in $Y_{\max}$ alone (from the same amount of electrons from thiosulfate oxidation in "*M. thiooxidans*", a 9% increase in $Y_{\max}$ was observed during growth on methanol, Chapter 5). In terms of Krebs' cycle, it has been shown that malate dehydrogenase is upregulated in the presence of DMS – possibly in addition to other enzymes not detected by the methods used in this study – suggesting that, rather than a simple oxidation of DMS providing electrons to the respiratory chain, a more complex system is in place causing upregulation of at least one step of Krebs' cycle in which reducing equivalents are generated. Given the magnitude of the increase in $Y_{\max}$ (and the relatively high m_S of this species when growing on succinate), it is likely that a proportion of the ATP generated is *via* the provision of reducing equivalents *and* electrons to the respiratory chain, rather than just electrons as would be the case in "*M. thiooxidans*".

In order to understand the role of malate dehydrogenase in the energy metabolism of *S. stellata*, metabolite quantitation (either using [$^{13}C_4$]-succinate and nuclear magnetic resonance spectroscopy or [$^{14}C_4$]-succinate and TLC-autoradiography and radiorespirometry) combined with enzyme assays of the full Krebs' cycle (and associated pathways) could provide data for metabolic flux analysis, which may help to identify the specific points of energy conservation in the presence of DMS.

CHAPTER 9

CONCLUDING REMARKS & PERSPECTIVES FOR FUTURE WORK

9.1 Conclusions

The key enzyme of DMS oxidation in *Hyphomicrobium* spp. – DMS monooxygenase - has been purified and characterised from *H. sulfonivorans*. DMS monooxygenase has been shown to be a two-component monooxygenase, related to bacterial luciferase, comprising two subunits – an $FMNH_2$-dependent DMS monooxygenase (DmoA) and an NADH-dependent FMN oxidoreductase (DmoB). For DMS, DMS monooxygenase from *H. sulfonivorans* has a V_{max} of 1250 nmol DMS oxidised min^{-1} (mg protein)$^{-1}$ and a k_M of 16.5μM, corresponding to a k_{CAT} of 5.2s^{-1}. EC 1.14.14.6 is proposed for DMS monooxygenase, as a distinct flavin-dependent monooxygenase. The enzyme has been shown to be inhibited by umbelliferone and heavy metals but not by methyl *tert*-butyl ether or chloroform, the classic inhibitors of DMS metabolism.

Chemolithoheterotrophic growth in which DMS carbon is assimilated to biomass whilst DMS sulfur is oxidised to tetrathionate with a net energy gain has been demonstrated in "*Methylophaga thiooxidans*". Both "internal" and "external" chemolithoheterotrophy has been observed in "*M. thiooxidans*", with endogenous and exogenous thiosulfate being oxidised to tetrathionate with a net energy gain. As far as can be found from the literature, this is the first recorded production of a polythionate from an organosulfur compound, as such, representing a potential new step in the biogeochemical sulfur cycle.

Stable-isotope probing with [$^{13}C_2$]-DMS has been performed for the first time and has confirmed *Methylophaga* spp. as dominant DMS-oxidising *Bacteria* in the marine environment. The oxidation of marine thiosulfate to tetrathionate has been demonstrating during a phytoplankton bloom, indicating that chemolithoheterotrophic *Bacteria* are active during the bloom.

Preliminary analyses have been carried out on the genome sequence of "*Methylophaga thiooxidans*" and the genes encoding the major enzymes of formaldehyde assimilation *via* the KDPG aldolase variant RuMP pathway have been identified. Genes encoding key enzymes involved in the dissimilation of methanol and methylated amines have been indentified, in addition to those involved in nitrogen uptake from ammonia, nitrate, nitrite and urea.

Chemoorganoheterotrophic growth, coupling the oxidation of DMS to DMSO with ATP formation, has been demonstrated in *Sagittula stellata* E-37^T, though the enzyme(s) responsible for this oxidation remain unclear.

9.2 Perspectives for future work

There is still a great deal of work to be done in terms of understanding the physiology and biochemistry of DMS-oxidising *Bacteria*.

With respect to the DMS monooxygenase from *H. sulfonivorans*, two key areas of work need to be done. Firstly, recombinant expression of the enzyme in *E. coli* needs to be investigated in order to obtain large amounts of pure protein for structural and further kinetic work. Secondly, the *dmoA* gene needs to be cloned from other organisms in order to be able to develop primers suitable for use on environmental samples or DNA obtained from SIP experiments.

With "*Methylophaga thiooxidans*", a genetic system needs to be established in order to be able to use mutagenesis-based methods to probe the proposed pathway of DMS oxidation. Attempts need to be made to purify and characterise the DMS demethylase and thiosulfate dehydrogenase. The organism is an excellent candidate for further work on thiophene metabolism and efforts need to be made to study the pathway of thiophene metabolism here. With a completed and annotated genome, it will be possible to use proteomic approaches to study the physiology of various compounds by "*M. thiooxidans*".

With *Sagittula stellata*, much work still needs to be done. The upregulation of malate dehydrogenase in the presence of DMS needs to be investigated and metabolic flux analysis and more detailed proteomics need to be performed in order to try and understand if this upregulation is caused by DMS or if it is an artefact of the energy-gaining process induced by DMS. The principle enzyme of DMS oxidation remains unknown in this species and this is an area that clearly needs more work.

Overall, more organisms need to be studied in pure culture and DMS oxidisers from culture collections need to be revisited in order to confirm the products of their DMS metabolism. Based on the current pathways of DMS oxidation that are known in *Bacteria*, the key enzymes that (the genes for which) could be used as functional probes for molecular ecological studies are DMS monooxygenase, MT oxidase and DMS demethylase. Efforts should be concentrated on purifying the latter two of these enzymes in order to understand their function and to obtain sequence data which can form the basis of efforts to clone the genes in order to begin the process of developing functional probes.

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APPENDIX:

SYNTHESIS OF SODIUM POLYTHIONATES

A.1 Sodium trithionate

180mL of a 6.7M sodium thiosulfate solution[17] was placed in a steel beaker in an ice bath and was stirred vigorously on a magnetic stirrer whilst maintaining a temperature of below 20°C. 280mL of “100 volume” hydrogen peroxide solution was added dropwise over 2 hours. Once the addition of peroxide was complete, the mixture was incubated (without stirring) on ice for 2 hours. Crystals of precipitated sodium sulfate were removed by filtration at the pump and were washed with 200mL ethanol. Filtrate and ethanol were placed in a 4-litre beaker and maintained at 3°C in an ice bath. To this mixture, 500mL ice-cold ethanol was added and the solution incubated at 0°C for 1 hour. Crystals of precipitated sodium sulfate were again removed by filtration at the pump and the precipitate washed with 200mL ethanol. Filtrate and washings were transferred to a 10L glass carboy containing 2L ice-cold ethanol and stirred at 4°C for 2 hours. Crystals of sodium trithionate were removed by filtration at the pump and were washed with 100mL ethanol and 100mL acetone. Crystals were dried *in vacuo* over silica gel. A yield of approximately 120g 98% pure sodium trithionate was obtained.

A.2 Sodium tetrathionate

500mL 4M sodium thiosulfate solution was placed in a 20L glass carboy, cooled to 4°C on ice with and stirred vigorously whilst 500g powdered iodine was added. To this solution, 1L 5.6M sodium acetate solution was added, followed by 8L ethanol. Crystals of precipitated sodium tetrathionate were recovered by filtration at the

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[17] For all polythionate syntheses, anhydrous sodium thiosulfate (≥ 98.0%) was used.

pump and were dissolved in 600mL 0.5M hydrochloric acid at 70°C. Iodine was removed by partitioning with *n*-hexane (approximately 2L in total) and sodium tetrathionate was recrystallised by filtering the solution into a beaker held at 1°C on ice. Crystals were washed in ethanol and dried *in vacuo* over silica gel. A yield of approximately 430g 100% pure sodium tetrathionate was obtained.

A.3 Sodium pentathionate

600mL 5M sodium thiosulfate solution was placed in a 5L beaker and 100mL of a 10% solution of arsenous acid in 12M sodium hydroxide solution was added. The mixture was stirred and cooled to -10°C in a 20% potassium chloride bath. 800mL 11.5M hydrochloric acid was added to precipitate sodium chloride, which was removed by filtration at the pump. The filtrate was incubated for 72 hours at 25°C and the precipitate of arsenic sulfides and elemental sulfur was removed by filtration at the pump. The filtrate was concentrated *in vacuo* at 40°C to approximately 200mL and was filtered to remove crystals of sodium chloride. 100mL glacial acetic acid was added to the filtrate, which was then cooled with stirring to -10°C, as before. 50mL glacial acetic acid was added to a solution of 67g sodium acetate in 250mL hot ethanol to produce a slurry. The slurry was added slowly to the cooled filtrate, which was maintained at -2°C in an ice-salt bath. A precipitate of sodium pentathionate was obtained after 15 minutes and was recovered by filtration at the pump before washing with absolute ethanol and recrystallising from 0.5M hydrochloric acid at 35°C and drying *in vacuo*. A yield of approximately 50g 100% pure sodium pentathionate was obtained.

A.4 Sodium hexathionate

300mL 7.6M hydrochloric acid was cooled to -35°C in a 62% (*w/w*) calcium chloride-ice bath. 80mL 5M sodium thiosulfate solution containing 9.7g sodium nitrite was added with shaking and incubated until a white precipitate of sodium chloride was produced. The mixture was then sparged with nitrogen for 30 minutes to remove nitrogen oxides and sulfur dioxide while maintaining a temperature of -35°C in a dry ice-water bath before removing the sodium chloride precipitate by filtration at the pump. The filtrate was concentrated *in vacuo* at 25°C to a thick paste of crude sodium hexathionate which was filtered through a sintered glass plate and washed with absolute ethanol before drying at 25°C overnight. The product was recrystallised from 2M hydrochloric acid at 80°C and washed with ether. A yield of approximately 10g 99% pure sodium hexathionate was obtained.

A.5 Sodium heptathionate

Sodium heptathionate was obtained by heating 2.5g sodium hexathionate (as prepared in 2.1.5.4) to 70°C in 3.5mL 2M hydrochloric acid and filtering to remove precipitated sulfur. The filtrate was cooled on ice and the precipitate of sodium heptathionate was recovered by filtration. Approximately 500mg sodium heptathionate at 90% purity were obtained (Wood & Kelly 1986).

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